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(54) Title: RAPID DETECTION AND IDENTIFICATION OF MICRO-ORGANISMS

(57) Abstract

The invention relates to the field microbiology, more specifically to the field of detection, identification and quantification or enumeration of micro-organisms. Micro-organisms, such as viruses, plasmids, bacteria, yeasts, fungi, algae, protozoa, plant or animal cells, and other prokaryotic or eukaryotic cells are in general unicellular organisms with dimensions beneath the limits of vision which thus escape easy detection. The invention provides methods and means for use *in situ* staining of micro-organisms comprising: a) mixing a material containing at least one micro-organism with a composition which can (partly) degrade a cell wall or cell membrane of a micro-organism thereby allowing for penetration through said wall and/or membrane of a (labelled) probe into said micro-organism, b) fixing said micro-organism with a fixative to retain its individual corpuscular character, c) reacting said probe with an antigen or nucleic acid molecule present in said micro-organism and d) detecting the presence of said probe in said micro-organism.

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Title: Rapid detection and identification of micro-organisms.

The invention relates to the field microbiology, more specifically to the field of detection, identification and quantification or enumeration of micro-organisms. Micro-organisms, such as viruses, plasmids, bacteria, yeasts, fungi, algae, protozoa, plant or animal cells, and other prokaryotic or eukaryotic cells are in general unicellular organisms with dimensions beneath the limits of vision which thus escape easy detection. Micro-organisms are omni-present, complex (mixed) populations can be present in materials as divers as (sea)water, bodily tissues or fluids, pharmaceutical preparations, foodstuffs, industrial products, waste, soil, plants, animals, other micro-organisms, and often their (specific) presence needs to be detected or monitored. Micro-organisms for example are widely used in fermentation processes, can be involved as pathogen in disease, and may be used in modern (recombinant) biotechnology; here, as elsewhere, it is essential to be able to properly identify and quantify the micro-organism(s) involved.

Traditionally, culturing techniques are used to establish the microbial status of a material. Culturing micro-organisms is an established way of isolating, detecting, identifying or quantifying them. Although a great number of micro-organisms can in general be cultured, propagated and manipulated in the laboratory, there are also micro-organisms for which culturing methods have not been developed or for which laboratory culture may be difficult if not impossible. For viruses, and other intra-cellular organisms, in general, cell-cultures are needed in which said organism grows to detect, identify or quantify the specific organism. Other micro-organisms can

be isolated and grown in various culture media under various, often very specific, conditions.

When taking starting material in which the presence of various different micro-organisms has to be detected by culturing, one generally needs to employ a diverse set of culture techniques. To be able to detect a specific organism, one has often to employ enrichment techniques, whereby the starting material is grown under conditions favouring growth of one specific (group of) organisms and suppressing growth of others. Identification of organisms by culturing, however, especially in the light of the need to distinguish several (closely) related organisms (such as bacterial strains), often requires prolonged culturing under a wide variety of conditions. Furthermore, organisms belonging to one specific strain may differ in that one has acquired additional genetic information, such as resistance to certain antibiotics, that is not present in another organism belonging to the same strain. Also, the micro-organism strains may differ in enzymatic activity. To detect such (acquired) characteristics, yet other culture techniques are then employed.

Quantifying the number of specific organisms present in starting material via culture techniques is relatively easy when it concerns starting material containing only one specific organism, e.g. in the case of monocultures. Such quantification can then be done using e.g. limited dilution tests. However, when in starting material that contains mixed populations of micro-organisms the identity and/or the relative presence or absolute numbers of various organisms need to be known, culturing gets often very cumbersome and tedious, and may even be impractical or impossible for several of the organisms present in the starting material, leading to biased results.

Alternative or additive techniques to culturing for the identification of micro-organisms in starting material can in principal be developed for those organisms for

which characteristics, such as specific antigenic or genomic information is known. Using antibody or nucleic acid probes specific for a micro-organism it is possible to identify such organisms in various materials. In *in situ* staining or detection, labelled probes are used that react with cell components. Probes such as specific antibodies, labelled directly or indirectly with reporter molecules (chromophores or enzymes and corresponding substrates) and used in e.g. immuno-cytochemistry, generally react with antigens (especially (poly)peptides or (glyco)proteins) that are specific for a certain micro-organism. (Labelled) nucleic acid probes or primers reactive with specific nucleic acids, such as genomic (ribosomal) or episomal genes, of the micro-organism find application in *in situ* hybridisation techniques, such as FISH (fluorescent *in situ* hybridisation) or in *in situ* amplification techniques such as *in situ* PCR (polymerase chain reaction). Detection of the labelled antibodies or probes or amplified sequences occurs generally by microscopic observation of the material tested. Cells are first immobilised on slides (fixed and lysed e.g. by drying, ethanol fixation, etc.) and subsequently reacted with antibody or nucleic acid (labelled) probes. Alternatively, cells can be kept in suspension until after the probe reaction, after which the suspension is filtered and the cells remain immobilised on a filter disc for analysis. Subsequent amplification of the probe signal allows detection of the labelled cells by microscope. Semi-automated systems, using digital image analysis and image intensification (image cytometric analysis) have been designed to help study microscopic preparations. However, image cytometry has the disadvantage that, in practical terms, at most some 500 to 1000 individual cells per microscopic preparation can be analysed. In general, a major drawback of above microscopic techniques is the limited sample throughput. The long analysis time required

limits the maximum throughput to 20 to 50 samples per day. Furthermore, although fixation and lysis of the cells in general suffices to immobilise antigens and nucleic acid to the cytoskeleton or the various organelles present in the cell, the cell in itself disintegrates and gets fragmented due to the fixation and lysis. As a consequence, those antigens and nucleic acids that are for example present (in solution) in the cytosol will disappear during fixation and lysis and cannot be detected. As a consequence, no insight can be gained by above microscopic techniques about (current) enzymatic and/or mRNA activity of the micro-organisms studied. In general, this basic problem in *in situ* staining using signal amplification systems relates to the diffusion of large-molecular weight molecules, such as enzymes, antibodies or (strept)avidin, into whole fixed cells. The permeabilization gets more difficult for larger molecules since the margin between the accesibility of target molecules and the loss of target molecules or complete cell lysis becomes very narrow (Amman et al., Microbiol. Rev. 59:143-169, 1995; Schonhuber et al, Appl. and Env. Microbiol. 63:3268-3273, 1997). Permeabilization of cells walls by pretreatment with lysozyme(EDTA) in general allow a restricted set of bacteria to be investigated, whereby it must be noted that lysozyme degradation often only permeabilises a minor fraction of the tested cells (causing heterogenous signals) and is in general only applicable with cells fixed on microscopic slides but not with cells in suspension.

Thus, techniques which allow rapid automated detection of the identity and number of a variety of micro-organisms present in the starting material are needed. Furthermore, techniques are needed which rapidly can give insight in the (enzymatic) activity of the micro-organisms involved. For example, rapid techniques to monitor the development of individual species or strains,

and/or their (current) enzymatic activity, involved in fermentation processes comprising complex mixtures of species would relay important information related to the management and control of such processes. Rapid flow cytometric techniques (such as FACSCAN) allow the recognition and even sorting of whole individual cells present in a solution; tenths of thousands of cells can be tested in one sample, sampling of testing material can be automated, and a multitude of samples can be tested daily. Rapid flow cytometric techniques have been applied in the detection of specific genes, nucleic acid sequences, antigens or proteins of individual eukaryotic cells, thus allowing rapid identification and quantification. However, a major problem to overcome when wanting to apply flow cytometry is maintaining sufficient integrity of the individual cells which need to be tested while at the same time making the content of cells accessible to the necessary probes. Fragmented or disintegrated cells can not be detected as an individual cell with flow cytometry; it is essential that the corpuscular character of the cell is maintained. There is a need for staining techniques allowing the detection of specific traits of individual whole cells in solution, which after all cannot be fixed and lysed to allow free access of (labelled) probes for *in situ* staining. Especially cells (such as most prokaryotic cells, plant cells, and such) surrounded by a cell wall or cell envelope have in general not been accessible to such techniques due to the relative impenetrable character of the cell envelope or cell wall surrounding these organisms, making it impossible for (labelled) probes to penetrate the cell and react with its specific antigen or nucleic acid. Furthermore, cells that are only surrounded by a cell membrane lyse easily, thereby loosing their corpuscular character. In addition, (labelled) probes are needed that can penetrate (the cell envelop) and cell membrane and can react specifically *in situ* with antigens

or nucleic acids contained in a (relatively) intact and unfixed or lysed, or not immobilised cell, and substrates are needed that can be used for *in situ* detection (e.g. via amplification) of the bound (labelled) probe present in such cells. Although a wide body of literature exists relating to FISH (for example EP 0497464, WO 97/05282, WO 96/36734, WO 96/34978, EP 0336412, EP 0422861, and others) these methods are in general only applicable for a limited number of, or even only one, species of micro-organisms, and do not allow for the detection of slowly growing or non-growing cells. Additionally, they for example do not, or only little allow detection of mRNA, and allow only little or no penetration of probes into the cell. In short, there is a need for techniques that can, while maintaining the corpuscular integrity of the majority of the treated cells, generate sufficiently large `holes' in cells to allow penetration of (labelled) probes and/or reporter molecules, which need is juxtaposed to a need for sufficiently small probes and reporter molecules that can penetrate relatively small `holes' that do not or barely jeopardise the cell's corpuscular integrity.

The invention provides a method for use in *in situ* staining of micro-organisms comprising: a) mixing a material containing at least one (fixed or non-fixed) micro-organism with a composition which can (partly) degrade a cell wall or cell membrane of a micro-organism thereby allowing for penetration through said wall and/or membrane of a (labelled) probe into said micro-organism, b) optionally fixing said micro-organism to further retain its individual corpuscular character, c) reacting said probe with an antigen or nucleic acid molecule present in said micro-organism, and d) detecting the presence of said probe in said micro-organism. A preferred embodiment of the method of the invention finds its application in *in situ* staining of bacteria, yeasts or fungi, or plant

cells, such as algae, or (obligatory) intracellular organisms, such as viruses, plasmids, and intracellular bacteria. An example of the method provided by the invention comprises treating a fluid containing a micro-organism with a composition which at least comprises one detergent, such as sodium taurocholate or other bile-salts or bi-polar lipid molecules, and at least one enzyme, such as a lipase and/or a carbohydrate degrading enzyme such as lysozyme, finizym, or mutanolysin. Yet another example is a method according to the invention wherein said composition at least comprises a proteinase, such as proteinase K and/or protease type XXI.

The method or permeabilization procedure represents a controlled process by which cells become more and more permeable to the probe such as a fluorophore upon prolonged incubation. At a certain point, lysis may start to occur resulting in diminishing fluorescence and loss of cells. Depending on the concentration of the reactants in the mixture and the incubation temperature, the first signs of permeabilization with a mixture according to the invention can be observed microscopically, allowing adjustment of said incubation parameters to the conditions desired.

Additionally, said composition may comprise at least one osmoticum, such as saccharose, sucrose, or lysine betaine, to regulate osmotic pressure and/or at least one cation, such as $Ca^{++}$ or $Li^{+}$ (but not significant amounts of $Mg^{++}$). Said composition may be optionally buffered, for example with Tris-HCl. Furthermore, the invention provides a method for *in situ* staining of micro-organisms wherein said micro-organism is fixed, to further retain its corpuscular character, with a fixative, for example a cross-linking agent, or selected from the group composed of (para)formaldehyde, glutaraldehyde, ethanol, methanol. A preferred embodiment of the invention is a method using a nucleic acid probe, for example an RNA or DNA-targeted deoxyribonucleic acid (DNA) (oligo)nucleotide probe, a ribonucleic acid (RNA) oligonucleotide probe, or a

peptide nucleic acid (PNA) probe. Nucleic acid probes used in the method of the invention ("*in situ* probes") are provided as well. As a consequence of the gentle, relatively non-denaturing, method provided by the invention most nucleic acid molecules in the cell are relatively little or not denatured, thereby greatly hampering hybridisation of most probes that otherwise would hybridise easily with stretches of denatured nucleic acid molecules. An example of such probes provided by the invention is given in Table 3. A further embodiment of the invention entails a method wherein said probe is labelled with a reporter molecule, for example a reporter molecule selected from any of the group of fluorochromes and enzymes. A preferred embodiment of the invention is a method wherein the reporter molecule is horseradish-peroxidase, preferably linked with a sulfo-SMCC linker to said probe. A further embodiment of a method provided by the invention is a method wherein the presence of said (bound) probe is detected by microscopy, image cytometry, fluorometry or flow cytometry, preferably be detecting fluorescence. A preferred embodiment of the invention is a method wherein the fluorescence of tyramine-fluorchromes is detected. A preferred embodiment of the invention is the use of a method of *in situ* staining provided by the invention wherein the micro-organism is not immobilized, for example to a slide or filter, but wherein detection of the stained micro-organism occurs in suspension. Also provided by the invention is a composition comprising a cell-wall degrading reagens for use in *in situ* staining. Such a cell wall degrading reagens comprises at least one detergent, such as sodium taurocholate and at least one enzyme, such as a lipase and/or aditionally comprises a carbohydrate degrading enzyme such as lysozyme, finizym, or mutanolysin. Additionally, said composition comprises at least one osmoticum, such as saccharose, sucrose, or lysine betaine, to regulate osmotic pressure and/or at least one cation, such as $Ca^{++}$ or $Li^{+}$ (but nor $Mg^{++}$). Said composition

may be optionally buffered, for example with Tris-HCl. Also provided by the invention is a nucleic acid probe linked with horseradish-peroxidase. Said probe and horseradish-peroxidase are preferably linked with a sulfo-SMCC linker. Also provided by the invention is a diagnostic test kit for use with *in situ* staining. Such a kit provided by the invention at least comprises a cell wall-degrading composition and/or nucleic acid probe provided by the invention, and can additionally at least comprise fixative, substrate, or buffer provided by the invention. The HRP-nucleic acid probe provided by the invention is also provided for use in other tests systems than *in situ* assays alone. Such a probe can for example be used in enzym-linked assays , such as a dip-stick but other formats are also possible, for the rapid detection of genes or gene-products in for example lysed bacterial samples. In such a format, the deposited reporter molecules can additionally serve as hapten for, e.g., anti-fluorescein-AP, antibodies via which an extra amplification step and the detection of very low numbers of target molecules is possible.

Of basic importance to all disciplines in micro-biology is the proper identification of the organisms at the various taxonomic (e.g. species) level. For example, in traditional (e.g., cheese-making) and modern (e.g. probiotics) biotechnological processes, techniques to monitor the development of individual species in complex mixed populations are of importance to the management and control of the process. Traditionally, this is performed by tedious cultivation techniques. More modern methods make use of ribosomal genes to identify micro-organisms (WO '91/00926). Although such molecular (ribosomal RNA-based) methods are suitable to identify micro-organisms directly under the microscope without cultivation these developments are still premature, and automated analysis is hampered by low signals and limited capacity of sample throughput. Often, only 70% of all the cells present in mixed natural populations can be

detected when universal 16S rRNA probes for fluorescence *in situ* hybridization are applied, even when digital image analysis and image intensification is used to collect fluorescence emission from microscopic preparations. Although image cytometric analysis is in general terms an appropriate tool for the extraction of morphological features or localisation parameters (e.g., as in genome-mapping of eukaryotic cells), signal amplification is ultimately desirable when natural mixed populations of bacteria (such as those occurring in seawater) are to be investigated. Image cytometry has the disadvantage that in practical terms, at the most, some 500 to 1,000 individual cells per microscopic preparation can be analysed. Furthermore, the long analysis time required limits the maximum throughput to about 50 samples per day. The relatively small number of samples that can be tested render the automation of the fluorescence *in situ* hybridization technique practically and economically unattainable. Flow cytometric analysis of samples would enable large scale analysis, if technically feasible. The present invention enables rapid flow cytometric acquisition of quantitative data from probe-labelled cells and the possibility to automate methods for *in situ* identification based on nucleic acid probes through generation of unequivocally high signals from such labels. Apart from the need to determine the taxonomic position (scientific name) of the micro-organism, the level of cellular (enzymatic) activity of cells is of considerable interest. The possibility to attach to individual cells not only a name but also an activity in a routine procedure cq. a practical protocol, will greatly advance microbiology. The detection of certain sub-populations with changed characteristics, i.e. that result in altered activity at the level of messenger RNA within complex fermenting populations is of great industrial interest. Identification of individual cells at the species level via ribosomal *in situ* probing does not always yield sufficient information on the characteristics of the mixed

population. The present invention provides a tool to measure the potential metabolic activity or the potential level of individual enzymes within individual cells via determination of the concentration of various messenger RNA's. Such determination optionally occurs in parallel to the procedure used for identification of a micro-organism with (16S, 23S) rRNA targeted DNA probes. The methods and means provided by the invention enables the simultaneous determination of name and activity. The rapid developments in the field of genetically modified and transgenic organisms, create an urgent need for methods to detect the modified or transgenic genes directly in the environment. Currently, molecular amplification, e.g. via PCR, of genes isolated from the environment give little information on the absolute quantity of such genes, their localisation, the identity or the numbers of the host organism. Large scale field trials with genetically modified micro-organisms can hardly be executed due to limited availability of methods to monitor the spreading of the organisms involved. In order to detect such genes, it is most appropriate to detect the expression of the genetic information within the organism. Currently, the most useful method would consist of monitoring specific messenger RNA's (mRNA's) in whole cells, i.e. without disrupting the natural situation. Powerful signal amplification methods are needed. Since the extend of enzymatic activity in organisms is largely determined by the amount of mRNA, a method to determine the amount of various mRNA-species in individual cells will prove extremely valuable for a wide variety of applications in biotechnology. Such a method has been developed for mammalian cells (WO 90/11523), but has never found wide application in microbiology due to the inaccessibility of micro-organisms to the probes and specifically to the size of the label used (50 kD horseradish-peroxidase). Mammalian cells are easily accessible to this label. However, bacteria, for example, have a rigid cell envelop consisting of among others

peptidoglycan and said method, when applied (Schonhuber et al., Appl. and Env. Micobiol 63, 3268-3273, 1997;Lebaron et al., Appl. and Env. Micobiol 63, 3274-3278, 1997) with peroxidase- or biotin-labelled probes, works in general only with well-defined target species, gives a signal amplification of only 7-20 fold compared with conventional fluorescence probes, is in general only applicable to cells fixed on microscopic slides and stains only a fraction of the cells, resulting in non-homogenous staining, again illustrating the need for permeabilization protocols also allowing successful *in situ* staining of cells in suspension. The present invention allows for controlled degradation of for example bacterial cell walls in combination with an amplification technique for the measurement of rRNA in micro-organisms thus allowing *in situ* staining and detection, for example by flow cytometric detection. The present invention provides a signal amplification of at least 30-50 fold, and even 80 fold, as compared with conventional fluorescent probes. The invention also provides a method to determine the amount of messenger RNA in micro-organisms and mammalian cells. The commercial impact of the any molecular analysis method stands or falls with possibilities to enlarge the scale and reduce the cost. For large scale analysis, the ability to automate methodologies is crucial. In the ideal case, a sample is taken, directly inserted into an instrument and the data are printed. The system that comes closest to this is *in situ* hybridisation (ISH), whereby the sample is treated with nucleic acid probes prior to insertion into the instrument. Quantification of the signal occurs preferably on a cell-by-cell basis. In order to automate analysis of whole bacterial cells, flow-cytometric detection is the most suitable instrumentation. The signals should for example therefore be fluorescent. Fluorescence in situ hybridisation (FISH) in combination with flow cytometry is possible with the invention here presented. Thus, fluorescent reporter molecules are needed to detect genes (DNA or RNA) or

transcripts (RNA) in cells. For any fluorescent labelling in mixed populations to be detected by flow cytometry and for it to be well separated from electronic background "noise-levels", cells must contain at least some 100.000 fluorescent molecules with highly fluorescent properties. This, in contrast to "normal" fluorescent microscopic detection for which an equivalent of some 10.000 fluorescent molecules is needed to reach detectable levels per bacterial cell in a mixed population. RNA's, the multi-copy transcripts of the DNA, are naturally amplified within each living cell, as such, they represent ideal targets for genetic labelling techniques. However, each bacterial cell contains some 20.000 identical ribosomal RNA (rRNA) molecules and some 200 identical messenger RNA (mRNA) molecules. If each of these molecules was tagged by one fluorochrome, signals would not be sufficient to automate the analysis. mRNA molecules represent the "coding" intermediates between DNA and proteins for which the genetic code is contained in the genome. The amount of mRNA that codes for a particular protein/enzyme indicates the level of expression of the gene and gives an impression of the potential quantity of available protein. Therefore, the quantity of a certain species of mRNA gives an impression of the potential enzymatic activity coded by that messenger. Messenger RNA *in situ* hybridization (mRNA-ISH) is possible as a result of the present invention. Traditional methods to determine the activity of enzymes are based on the formation of product or disappearance of substrate in an *in vitro* test-system. Alternatively, antibodies or fragments of these can be produced that specifically recognise certain proteins. When such antibodies are fluorescently labelled, the amount of fluorescence produced is proportional to the amount of protein present. Such assays can also be performed in *in vitro* test-systems. They can, however, not be performed *in situ* in prokaryotic micro-organisms, due to the inaccessibility of the bacterial cell-wall to the (large) antibody molecule. Measurement of mRNA's, as enabled with the

methods and means provided by the invention with *in situ* protocols, will boost research and biotechnology in general due to the possibility for on the spot detection of the presence of certain species of bacteria combined with a determination of their activity. This also creates the possibility to differentiate between individual strains of (bacterial) species. Traditional methods to determine the composition of mixed microbial populations require cultivation on selective and non-selective media and the subsequent identification of the obtained isolates. These methods are laborious, time-consuming and prone to statistical and methodological error. For this reason, the dynamics of the population composition are very difficult to monitor. Furthermore, the detection of specific genotypes, containing useful or harmful genes, can not be performed *in situ*. In industry, specifically the food, fermentation, and pharmaceutical industry comprising classic or modern (recombinant) biotechnology, monitoring of for example bacteria- or yeast-dependent fermentation processes and the genetic composition and activity of the fermenting population is essential for quality assurance for example with respect to hygiene, product control, process control and management, culture stability, and so on. The invention provides means and methods to exert better control and management of such cultures. For example, in modern biotechnology, heterologous (over)expression of proteins from genes foreign to the cultured organism is often hampered by regulation difficulties. Although for example inducable expression of genes governed by inducable promotors or other regulators becomes more and more applied, the exact moment in time to up- or down-regulate expression by regulating promotor activity is hard to assess. The invention provides means and methods to study mRNA (e.g. of foreign genes) activity in such fermentation cultures, thereby allowing better control. Environmentally, monitoring of complex mixed bacterial populations is elemental in predicting the effectiveness of

bioremediation and gas- and wastewater-treatment. Soil microbiologists are in need for tools that explain nutrient regeneration potentials in terms of biomass of individual metabolic clusters of bacteria.

Hybridisation probes in combination with PCR-amplification of target nucleic acid, are often applied for the detection of specific bacterial taxa or specific bacterial genes in mixed populations. Currently, by using PCR techniques the qualitative presence of a single cell against a background of $10^9$ non-target cells can be confirmed. As such the technique is extremely sensitive. However, the technique gives little or no quantitative information on absolute cell numbers, or the distribution of the gene over the total population. Fluorescence *in situ* hybridisation alllows, contrary to PCR, enumeration of individual cells that belong to a certain bacterial group, or the demonstration of particular genes *in situ*. However, in general the obtained fluorescence signals are too low to perform high-speed automated detections and the technique is too insensitive to allow the detection of single genes in individual cells. A key factor to this problem is the fact that oligonucleotide probes can be constructed with only a limited number of fluorochromes (maximum of 3). When nucleic acid probes could be combined with appropriate enzymatic in stead of fluorescent labels or reporter molecules and when these enzymatic labels would be able to synthesize hundreds of fluorescent product molecules it would be possible to identify single cells *in situ*, even if these cells were having low amounts of target material, to detect single genes in individual cells, and to detect messenger RNA species *in situ* in whole cells. A prerequisite would be that these fluorescent molecules stay in the cell. However, available enzyme/substrate combinations produce water soluble fluorescent molecules that diffuse out of the cell. Tyramides, however, are transformed into tyramide-radicals by horseradish peroxidase (HRP). The tendency of such radicals

to react and bind to anything in their vicinity, thereby remaining inside the cell, makes them ideally suited as fluorochrome carriers. However, these can not be used because suitable nucleic acid probes linked to horseradish-peroxidase are not available. Alternative strategies, using hapten(e.g. biotin)-labeled oligonucleotide probes which can subsequently be detected by HRP-labelled streptavidines are routinely used in human chromosome mapping and *in situ* hybridisation of mammalian cells. However, without exception, these molecules are too bulky too penetrate a (bacterial) outer cell wall (cell envelop). With the combination of fluorescent tyramides and HRP-labelled probes, individual genes (liberated from fragmented cells) can be detected by FISH(WO 90/11523). However, the probe-HRP complex is too large to freely enter bacterial cells. Therefore, the invention provides specific protocols, means and methods that are needed to permeabilize the (bacterial) cell wall. Furthermore, the invention provides a method to couple the HRP molecule directly to the nucleic acid probe via a $C_6$-thiol linker. Such a nucleic acid probe is derived from a thiolated probe linked to horseradish-peroxidase with a sulfo-SMCC linker, or a linker equivalent thereto. The invention herewith provides a method for linking a nucleic acid probe to horseradish peroxidase comprising mixing a thiol oligonucleotide with a SMCC-HRP complex, incubating said mixture and purifying it by ultrafiltration. With such a labelled probe provided by the invention, the invention provides a probe molecule which is small enough to penetrate a cell wall when this barrier is properly and controllably degraded. In order to control cell wall degradation, various cell wall degrading reagents and compositions are provided by the invention that allow the penetration of a HRP enzyme linked to an oligonucleotide by a C6-thiol linker into bacterial cells. In order to obtain high levels of fluorescence, tyramide-fluorochromes are constructed that act as substrate for the HRP. The resulting tyramide-fluorochrome radicals attach randomly in the cell

whereby fluorescence is attained for very long periods (up to months). The methods and means provided by the invention allow for example the direct detection of specific bacterial species (through rRNA-probing) in environmental, clinical or industrial samples via microscopic observation, automated image analysis or flow cytometric methods. Also, potential enzymatic activity determined by the quantification of mRNA species can be measured *in situ* in whole cells. Also, the method and means provided by the invention allow the detection of single genes within individual bacterial cells by using microscopic (image) analysis or flow cytometry. The invention allows for the use of any fluorochrome (fluorescein, rhodamine, coumarine, etc.) that can be amino-esterified. This enables the use of multiple-colors, and thus the detection of various parameters simultaneously. At present, measuring mRNA in whole bacterial cells by *in situ* hybridisation to detect expression of certain genes is cumbersome, when not using the methods and means provided by the invention. The reported method uses large multiple-labelled transcript probes (some 200 nucleotides long) instead of the small synthetic oligonucleotide probes and is not of great commercial interest since the production of the large transcript-probe used is very tedious and cannot be performed on a routine basis. The measurement of mRNA in mammalian cells is a known and commercially available technique (ELF™, Molecular Probes, Eugene, USA). In ELF™ a fluorescent precipitate is formed that will not diffuse away. However, this methodology cannot be used for bacteria since the necessary enzyme (alkaline phosphatase) is more than twice as large as peroxidase and cannot enter the bacterial cell wall without significant damage to cellular integrity and morphology. The method linking HRP to nucleic acid probes as is provided by the invention can be used to label for example peptide nucleic acid (PNA) -probes as well as oligonucleotide-probes. By using PNA-probes, hybridisation can occur in the absence of NaCl which allows for

simultaneous use of antibodies during hybridisation. Furthermore, the rate of hybridisation is increased several fold by using PNA's in stead of DNA's.

The methods and means provided by the invention can be used to screen large amounts of samples for the presence of groups/genera/species/subspecies of micro-organisms by flow cytometry and microscopy and to count the absolute number of cells that belong to these groups. Furthermore, determination of the absolute level of fluorescence allows for quantification of ribosomal content of the cells, even when very low levels of ribosomes are present such as in slowly growing bacteria (oligotrophic bacteria and mycobacterium species). ITS-targets, available in low-copy numbers, can be targeted, which allows the detection of bacteria down to the strain-level.

The methods and means provided by invention can be used to screen large amounts of samples for the presence and enumeration of cells that express specific mRNA's. The quantification of the fluorescence derived from an mRNA probe provided by the invention used for this purpose allows the determination of the exact level of expression of genes coding for e.g., toxins, metabolic enzymes, excretory proteins, anti-microbial substances like antibiotics or bacteriocines, lytic enzymes, (exo-)polysaccharides, membrane transport proteins. Quantification of the total mRNA-pool by using a poly-T oligonucleotide approach will enable the post-mortem determination of the physiological state of bacteria. This is a long-standing issue in microbiology.

The methods and means provided by the invention can be used to screen large amounts of samples for the presence and enumeration of cells that harbour specific (foreign) genes, gene-constructs, viruses, plasmids, and so on. The quantification of the fluorescence derived from a probe provided by the invention used for this purpose allows for the quantification of chromosomal and extra-chromosomal genes, plasmids or gene-constructs that code for e.g.,

vaccines delivered by micro-organisms, toxins, metabolic enzymes, excretory proteins, anti-microbial substances like antibiotics or bacteriocines, lytic enzymes, (exo-)polysaccharides, membrane transport proteins, food preservatives, enzymes, and so on. The methods and means provided by the invention can be used to screen large amounts of samples for the presence of cells that harbour bacteriophage or virus.

The HRP-nucleic acid probe provided by the invention can also be used in other tests systems than *in situ* assays alone. Such a probe can for example be used in enzym-linked assays , such as a dip-stick but other formats are also possible, for the rapid detection of genes or gene-products in lysed bacterial samples. In such a format, the deposited reporter molecules can additionally serve as hapten for, e.g., anti-fluorescein-AP, antibodies via which an extra amplification step and the detection of very low numbers of target molecules is possible.

The methods and means provided by the invention find application for example as diagnostic kit in the broad field of microbiology. A (non-extensive) list may include several possibilities, such as: To study the effect of novel foods (prebiotics), probiotics, and functional foods on the intestinal microflora of both man and animal. To monitor the development of mixed bacterial populations that are essential for industrial fermentation processes in classical (cheese and other dairy fermentation, beer, wine, etc.) as well as in modern biotechnology (heterologous (over)expression of proteins, etc.). To rapidly detect microbial contamination in product or production tools from raw material to end product (hygiene control). To identify and quantify the presence and activity of micro-organisms in wastewater treatment systems. To identify and quantify the presence and activity of micro-organisms in bioremediation processes. To determine the microbial status of (polluted) soils, waters, gasses, etc. To determine the status of potable water, swimming water and

surface water with respect to pathogenic bacteria and human health. To study the effect of antibiotics on the intestinal microflora of both man and animal. To study the effect of registered and novel medicines on the intestinal microflora of both man and animal. To perform diagnostics/rapid detection of pathogenic infections in man and animal including livestock. To detect bacterial resistance to antibiotics. To study the effect of homeopathic preparations and reform products on the intestinal microflora of both man and animal. To rapidly detect microbial contamination in product or production tools from raw material to end product (hygiene control). To detect specific "caries-generating" oral streptococci for the toothpaste industry. To detect pathogens or consortia of bacteria that are detrimental to crop development, yield or quality during production, harvest, storage and transport.

The permeabilization procedure allows for the first time the performance of *in situ* hybridization experiments with all of the bacteria which contain rigid polysaccharide capsules, extensive peptidoglycan layers, extraordinary cross-linking moieties in their peptidoglycan layer and/or that can prevail in the form of impermeable spores. Cells need not be brought to the vegetative state prior to *in situ* hybridization. The invention allows for the first time the direct detection of non-growing cells or cells obtained directly from the natural environment by *in situ* hybridization. By combining enzymatic labeling of oligonucleotides and methods to allow the entering of such conjugates into the cells as well as the formation of fluorescent precipitates, the present method ensures sufficient signal amplification as to yield fluorescence signals strong enough to allow the *in situ* detection by fluorescence in situ hybridization of low copy numbers of rRNA molecules. Thereby, the present invention provides a methodology to detect, e.g., *Mycobacterium tuberculosis* directly in sputum samples or bacteria obtained

from soil or bacteria obtained from watersamples by *in situ* hybridization. Such cells normally contain only 50-100 ribosomes and can not be detected by conventional fluorescence *in situ* hybridization.

The invention allows for the first time the direct detection of messenger RNA's by *in situ* hybridization. By combining enzymatic labeling of oligonucleotides and methods to allow the entering of such conjugates into the cells as well as the formation of fluorescent precipitates, the present method ensures sufficient signal amplification as to yield fluorescence signals strong enough to allow the *in situ* detection by fluorescence in situ hybridization of the naturally (very) low copy numbers of mRNA. Therefore, the present invention provides a methodology to detect, e.g., protease gene expression in Lactococcus lactis cells by *in situ* hybridization. Such cells normally contain only 50-100 mRNA molecules that can not be detected by conventional fluorescence *in situ* hybridization.

The invention allows for the first time the direct detection of streptococci by *in situ* hybridization. The present method provides for a permeabilization formula which can permeabilize streptococcal cell-walls without the need of prior formaldehyde fixation. The present invention thereby provides a methodology to detect, e.g., streptococci by *in situ* hybridization. Such cells can not be detected by conventional fluorescence *in situ* hybridization techniques. The invention allows for the first time the quantitative measurement of cells stained by *in situ* hybridization on a flow cytometer. The present method provides for a methodology with which cell lysis is prevented. Due to such a prevention, post-hybridization washing of cells is now effective (no background signal) and cells can be measured quantitatively by using methods such as flow cytometry.

The invention provides for the first time an optimized methodology of *in situ* hybridization staining by which cells

from natural samples, such as occur in feacal flora, can be measured on a flow cytometer. The present method provides for a methodology in which the pH of the buffer is adjusted such that FISH-derived fluorescence is optimal. Due to such an optimization, post-hybridization fluorescence is high enough to ensure that cells can be measured quantitatively by using methods such as flow cytometry.

As another embodiment, the invention describes a washing procedure that allows an effective post-hybridization washing of hybridized cells while maintaining sufficient cell density for flow cytometric measurement.

The present invention provides for a permeabilization formula that allows for the first time the performance of *in situ* hybridization experiments with all of the bacteria which either contain rigid polysaccharide capsules, extensive peptidoglycan layers, extraordinary cross-linking moieties in their peptidoglycan layer and/or that can prevail in the form of impermeable spores and that breaks down all structural cell wall components in a controlled fashion. One of the embodiments of the present invention is a permeabilization formula, another embodiment of the present invention is a signal amplification methodology which, when used in combination ensure i) the formation of pores or gaps in the cell wall of sufficient size as to facilitates the entering of bulky molecules such as horseradish peroxidase enzymes conjugated to oligonucleotides and ii) that facilitates the formation through such molecules of sequence-specific fluorescent precipitates thus allowing the microscopic or flow cytometric detection of cells stained by fluorescence in situ hybridization.

The invention is further explained in several examples which can not be seen as limiting the invention.

**Examples**

Example 1 Cell wall-degrading composition

The consecutive and combined action of several enzymes and chemicals have a degrading activity on the cell wall. A combination of enzymes results in the systematic - and thus controllable - degradation of the cell-envelop of Gram-negative and Gram-positive bacteria. These envelopes consist of a number of distinct layers, each with their own set of biomolecules. A biochemical background and information on resistance of these structures to probe penetration are presented in table 1. A function of the various components of a permeabilisation or degrading composition or cells wall degrading reagens is presented in tabel 2.

*Example of a possible preparation of a degrading composition (10.0 ml, sufficient for 50 reactions)*

Solution A: 20 ml 48% (w.v) sucrose solution (9.6 g sucrose in 20 ml of Milli-Q water); sterilize by autoclaving;

Solution B: 1 ml 1% (w/v) sodium taurocholate solution (10 mg (Na)taurocholate in 1 ml of sterilized Milli-Q water);

Solution C: 1 ml 11.1 % (w/v) calcium chloride solution (11.1 mg $CaCl_2$ in 1 ml of Milli-Q water); sterilize by autoclaving;

Solution D: 100 ml 1M Tris-HCl, pH 7.5 solution (12.1 g Tris(hydroxymethyl)-aminomethane in 90 ml of Milli-Q water, add conc. HCl to pH 7.5, add Milli-Q water to 100 ml); sterilize by autoclaving;

Solution E: 1 ml 20% (w/v) lysozym solution (200 mg lysozym in 1 ml of sterilized Milli-Q water);

Solution F: 1 ml 1% (w/v) lipase (10 mg of lipase in 1 ml of sterilized Milli-Q water);

Solution G: finizym, no preparations needed; purchase commercially from Novo Nordisk France;

Solution H: 1 ml 10.000 U/ml mutanolysin (10.000 U mutanolysin in 1 ml of sterilized Milli-Q water);

Mix according to the following formula: 2,730 ml solution A; 0.234 ml solution B; 0.390 ml solution C; 0.195 ml solution D; 0.117 ml solution E; 0.021 ml solution F; 0.042 ml solution G; 0.047 ml solution H. Filter over 0.2 µm membrane filter, transfer to 15 ml polypropylene bottle, freeze to -80°C and freeze-dry to powder for 48 h. Before use in permeabilization protocols reconstitute the lyophilized powder with 9.2 ml of sterilized Milli-Q water. Use 0.2 ml of the reconstituted and cooled (4°C) formula to treat a cell-pellet of $10^7$-$10^9$ cells. Incubate the cells for 1-15 min at 4°C in the permeabilization formula.

*Cell permeabilization:*

Example: Lactococcus lactis cells in LD-Bos cheese starter culture.

Clear a LD-Bos cheese starter culture by mixing 1 ml of culture with 30 ml of 0.2% EDTA. Incubate for 30 min on a roller-bench. Pellet the cells by centrifugation for 10 min at 10.000 rpm. Wash the cell pellet in 15 ml of PBS (1000 ml of Milli-Q water, 8 g of NaCl, 0.2 g of KCl, 1.81 g $Na_2HPO_4*2H_2O$ and 0.24 g of $KH_2PO_4$, pH 7.4). Pellet the cells by centrifugation for 10 min at 10.000 rpm. Resuspend the pellet in 4 ml of 4% paraformaldehyde (4 g of paraformaldehyde; 0.8 g NaCl; 20 mg KCl; 180 mg $Na_2HPO_4*2H_2O$ and 24 mg of $KH_2PO_4$, pH 7.4). Incubate for 10 min at 4°C. Dilute 400 µl of the fixed cell suspension in 1100 µl of PBS, wash 3 times by successive centrifugation and resuspension of the cells in 1.5 ml of PBS. During washings, include a short (5 min) incubation in each newly

applied PBS solution. This will allow for the complete removal of intracellularly accumulated formaldehyde. Resuspend the washed cell pellet in 200 µl of ice-cold permeabilization formula and incubate for 15 min at 4°C. Stop the treatment by adding 500 µl of 4% paraformaldehyde and incubate for 30 min at 4°C. Add 800 µl of PBS and wash 3 times by successive centrifugation and resuspension of the cells in 1.5 ml of PBS. Resuspend the washed cell pellet in 500 µl of PBS:ethanol (1:1). The thus treated cells of the LD-Bos cheese starter culture can be used for in situ hybridization with rRNA, mRNA or DNA-targeted deoxyribonucleic acid (DNA) oligonucleotide probes, ribonucleic acid (RNA) oligonucleotide probes, peptide nucleic acid (PNA) probes that are for their subsequent detection labelled with reporter molecules such as:

- fluorescent labels (e.g., fluorescein, rhodamine, methylcoumarine, tertramethylrhodamine, aminomethylcoumarin acetic acid, bimane, ethidium, europium [iii] cryptate, La Jolla blue, nitrobenzofuran, pyrene butyrate, terbium chelate, texas red, or cyanine dyes such as Cy3, Cy5 or Cy7);
- enzymatic labels (e.g., oxidases, peroxidases, phosphatases, luciferases, dehydrogenases, hexokinases or papain);
- enzyme inhibitors (e.g., phosphonic acid)
- proteinaceous labels (e.g., streptavidin, antibodies, Fab fragments or aequorin);
- chemiluminescent labels (e.g., acridinium esters, isoluminol, luminol or ruthenium *tris*[bipyridyl])
- hapten (e.g., biotin, digoxigenin, ethidium, glucosyl, sulfone, either
- metal complexes (e.g., 1,4-diaminoethane platinum complex)
- miscelaneous labels (e.g., small latex particles, poly AMP, pyrene)

- phosphors (e.g., Yttriumoxisulfide plus europium or zinc silicate plus arsenic and manganese)

Furthermore, the thus treated cells are permeable to large biomolecules and the procedure can be used to introduce large (bio)molecules of any kind in the bacterial cell. The procedure can be shortened by combining separate successive steps, e.g., (para)formaldehyde fixation can be combined with ethanol dehydration in one step. Furthermore, the (bio)chemicals in the protocol can be substituted for any (bio)chemical with comparable activity, e.g., ethanol can be replaced by methanol or propanol and the enzymes can be changed for enzymes with comparable biological catalytic activity provided that the environment of the reaction is modified according to routine skills to suit the new (bio)chemistry.

Example 2 5'-Horseradish peroxidase labelling of synthetic oligonucleotides

*Step 1. De-protection of commercially derived 5'-thiol labelled oligonucleotides:*

Deoxyoligonucleotides (10-30 mers) with a protected thiol (SH) group attached to the 5-prime end via a C-6 spacer arm were commercially derived (Eurogentec, Seraing, Belgium). The thiol-groups were de-protected by incubating the oligonucleotide (2 mg/ml) in DP buffer (50 mM (Na)phosphate, pH 7.4; 5 mM EDTA; 100 mM NaCl; 50 mM hydroxylamine-HCl) for 30 min at 30°C.

*Step 2. Linking of the horseradish peroxidase enzyme to a sulfo-SMCC linker:*

Solution 1: 50 mg/ml of sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate; Pierce, Rockford, IL, USA) in DMSO (dimethylsulfoxide).

Solution 2: 10 mg/ml of enzyme (HRP; Sigma Chemical Corp., St. Louis, USA) in PEN buffer, pH 8.0

(50 mM (Na)phosphate, pH 8.0; 5 mM EDTA; 100 mM NaCl).

A volume of 10 µl of freshly prepared solution 1 was mixed with 200 µl of solution 2 and the mixture was incubated at 30°C for 1 h. After incubation, 790 µl of PEN buffer, pH 6.7 (50 mM (Na)phosphate, pH 6.7; 5 mM EDTA; 100 mM NaCl) was added and the SMCC-HRP complex was purified via ultrafiltration using Centricon 30 (Amicon, Beverly, MA, USA). The complex was washed 3 times with PEN buffer, pH 6.7 in Centricon 30 ultrafilters and the supernatant of the final wash was made up to 200 µl with PEN buffer, pH 6.7.

*Step 3. Cross-linking of the HRP-SMCC complex to the de-protected oligonucleotide-thiol:*

A volume of 67 µl of the deprotected thiol oligonucleotide (step 1) was mixed with 200 µl of the purified SMCC-HRP complex (step 2) and the mixture was incubated at 30°C for 1.5 h. The cross-linked enzyme/oligonucleotide complex was purified via ultrafiltration using Centricon 30 ultrafilters. The complex was washed 3 times with PEN buffer, pH 6.7 in Centricon 30 ultrafilters and the supernatant of the final wash was made up to 67 µl with PEN buffer, pH 6.7. The enzyme-labelled probes were stored at 4°C. For hybridization, the probe-solution was diluted 10 to 100 times in PEN buffer, pH 6.7.

The (bio)chemicals in the protocol can be substituted for any (bio)chemical with comparable activity, e.g., the cross-linker sulfo-SMCC can be replaced by any cross-linker (such as sulfo-GMBS or DTBP) provided that the environment of the reaction is appropriately modified to suit the new chemistry. Examples of such linkers are

succinimidyl-, salicylamido- or hydrazide- derivatives that contain at least two reactive sites that are homobifunctional or heterobifunctional reactive towards (-NH2) amines
[e.g., 1,5-Difluoro-2,4-dinitrobenzene (DFDNB), Dimethyl adipimidate 2HC1 (DMA); or Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC)]
(-SH) sulfhydryls
[e.g., 3-[2-Pyridyldithio]propionyl hydrazide (PDPH); 1,4-Di-[3'-(2'-pyridyldithio)-propionamido)]butane (DPDPB); or Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC)];
carbohydrates
[e.g., Azidobenzoyl Hydrazide (ABH)]; or
(-COOH) carboxyls
[e.g., 4-[*p*-Azidosalicylamido]butylamine (ASBA) or 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide Hydrochloride (EDC);
or that contain one non-selective photoreactive cross-linking site and one of the selective reacting sites mentioned above.

Methodologies for preparing enzyme labelled probes are known in the art. The use of SMCC linkers for this purpose that activate proteins or enzymes to a thiol function are also known in the art. (e.g. **Ruth, J.L.** 1994. Oligonucleotide-Enzyme conjugates. *In:* Protocols for oligonucleotide conjugates. (Agrawal, S., ed.). *Methods in Molecular Biology* 26:167-185; **Alul, R.** 1993. Chemical synthesis of DNA and DNA Analogs. *In:* DNA probes. Pp. 69-136. (Keller and Manak, eds.). 2nd ed. Macmillan Publ. Basingstoke, UK.). However, the methods by which such linkers are used and the methods by which enzymes are attached to thiolated oligonucleotides is impracticle. Without exception, protocols that are known are

based on chromatographic purification of reaction products and require the collection of serially eluted fractions from purification columns. For example, the methodology proposed by Garman (EP 0422861A) first requires the purification of the maleimido derivatised HRP by a NAP 25 desalting column and collection of a 1.6 ml fraction (Example 8). Subsequently, the purification of enzyme-labeled oligonucleotide (example 9) requires the purification of the thiolated oligonucleotide over a NAP 5 desalting column, the mixing of the thus thiolated and purified oligonucleotide with the maleimido-derivatised HRP and finally the purification of the HRP-labeled oligonucleotide over a Biogel P-100 column. Such a methodology is laborious, does not ensure high yields of functional product, and requires experience with column chromatography by the user. Finally, it is not ruled out that eluted functional products are missed in the collection process, or that collected fractions contain no product, or that collected fractions contain product of insufficient yield.

The invention provides a method which employs a heterobifunctional linker, such as SMCC, in a specific protocol which is both rapid, yielding a constant product, not requiring the use of post-purification yield measurements or the identification of the specific product-containing eluate fractions. The current invention provides a methodology that is so simple that it can be based on the use of ultra-filtration devices (filter-columns) in which the reaction products are retained and of which the filtrate contains the non-desired reactants such as unlabeled probes or non-reacted linkers. The invention provides as a part of the invention a protocol for attaching an enzyme via a linker to an oligonucleotide in a two-step method without the need of post-synthesis purification and without the need of incorporating post-purification yield controls. This new method is highly efficient, highly reproducible and excludes

the possibility of losing the product or retrieving non-functional products (unlabeled probes). As one end-product, the present method generates enzyme-labeled oligonucleotides (reaction product A) which can immediately be used in *in situ* hybridization experiments. As non-functional end-products, the present method may yield un-reacted enzyme (reaction product B) as well as enzyme with a SMCC moiety attached to it (reaction product C). Reaction products B and C need not be purified from reaction product A as both B and C are non-functional in *in situ* hybridization experiments and do not interfere with the in *in situ* hybridization experiments. Therefore, the purification of fractions B and C from the end-product pool would represent unnecessary steps and would only complicate the procedure. The present invention therefore represents an important improvement of the art. The proposed method for preparing the HRP-labeled oligonucleotides is therefore one of the embodiments of the present invention.

Example 3. HRP-Labelling and use of Fluoresceine-Tyramide in whole cell fluorescence in situ hybridization with Lactococcus lactis cells.

*Fluoresceine-Tyramide (FT) substrate.*

Tyramine (Sigma) was linked to Fluoresceine-NHS (Boehringer) by mixing 15 mg of fluoresceine-NHS in 200 µl DMSO with 150 µl tyramine (20 mg/ml $H_2O$) and using a 4 h incubation at 37°C. The resulting complex was diluted 10 x in DMSO (final concentration 7 mM).

*5'-HRP labelling of synthetic oligonucleotides.*

Commercially derived deoxyoligonucleotides with 5'-$C_6$-thiol (SH) were deprotected with hydroxylamine in PEN buffer (50 mM (Na)phosphate, pH 7.4; 100 mM NaCl; 5 mM EDTA) for 30 min at 30°C. This modified oligonucleotide was subsequently cross-linked to a horse radish peroxidase (HRP)-Sulfo-SMCC (Pierce)

complex at pH 6.7 for 90 min at 30°C. The HRP-SMCC complex was formed by incubation of 10 mg/ml HRP with 2.5 mg/ml SMCC for 1 h at pH 8.0 and 30°C.

*Cell wall degradation and storage*

Cells of *Lactococcus lactis* subsp. *cremoris* were grown in overnight culture. Cells from two ml of culture were washed in PBS and resuspended in cell wall degrading reagens (50 mM Tris-HCl, pH 7.0; 0.03% (w/v) Na-taurocholate; 5 mM $CaCl_2$, 20% (w/v) sucrose; 0.5% (w/v) lysozym; 0.003% (w/v) pancreatic lipase; 1% v/v finizym. Cells were incubated at 37°C for 30min, washed in PBS (0.15 M NaCl; 2.5 mM KCl; 10 mM $Na_2HPO_4$, pH 7.4) and fixed for 2 h in 4% (w/v) paraformaldehyde. Fixed cells were washed twice in PBS and resuspended in a mixture of PBS and ethanol (96%) (1 : 1). Cells were stored at -20 °C for at least 1 h prior to hybridisation.

*Hybridisation*

Hybridisation was carried out by mixing 10 µl of the cell suspension in ethanol with 90 µl of hybridisation solution (20 mM Tris-HCl, pH 7.2; 0.9 M NaCl; 0.1% SDS; 0.48 M ureum; 0.25 ng/ml HRP-labelled oligonucleotide probe). Cells were hybridized for 16 h at 37 °C.

*Probe detection through HRP catalysed reporter deposition.*

After hybridisation, cells were washed 4x with 1/10 strength hybridisation buffer. Cells were incubated in fluoresceine-tyramide (FT) substrate solution diluted in PER buffer (0.1 M Tris-HCl, pH 8.5; 10 mM imidazol; 0.01% $H_2O_2$) for 30min at RT. After the final washing step in washing solution (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% v/v Tween 20) cells were either used immediately for flow cytometric analysis or were filtered onto a 0.2 mm pore-size Isopore™ polycarbonate membrane filter (Millipore Corporation, U.S.A.) for epifluorescence microcopic observation. Filters were mounted

on microscope slides with Vectashield™. Hybridised cells were viewed under blue light excitation using an Olympus BX40 fluorescence microscope using epi-illumination. Flow cytometric analysis occurred at 488 nm by using a Becton Dickinson FACScalibur equipped with a 15 mW argon ion laser and a 10 mW red diode laser. TOTO I (Molecular probes Europe BV, Leiden, the Netherlands) was used as the counter stain (figure 1).

Example 4. Detection of protease mRNA gene-transcripts in Lactococcus lactis spp. cremoris

*Culture and growth conditions.*

Two strains of L. lactis spp. cremoris str. MG1363 (NCDO 736), one which was protease deficient (Prt-) and one in which the defect was cured (Prt+) were grown in M-17 broth and in skim-milk at 30 °C. Every 30 min, 1 ml samples were withdrawn aseptically from all four cultures and these samples were immediately mixed with 100 µl of 37% paraformaldehyde. Samples were stored at 4°C until analysis.

*Clarification of milk and preparation of microscope slides*

100 µl of the fixed skim-milk samples was diluted into 1 ml of 10M EDTA (pH 12.0) and incubated at room temperature for 1 h. Samples were centrifuged at low speed (100 x g) and the supernatant was collected. Ten µl of this cell-suspension was spread onto a microscope slide and dried at 40°C for 30 min. After incubation with 20 µl of cell-wall degrading reagens for 15 min at 4°C, cells were hybridized with protease-specific DNA-probes for 16 h at 45°C.

*Protease-specific DNA probes.*

Three protease specific probes were developed and tested in this test-system: PRT1245 (5'-gcccctaaagcgactgtacc-3'), PRT4548 (5'-gataccgccgccgttcaca-3') and PRT6497 (5'-tgtcagacgcatcagcactg-3'). All three probes were 5'end labelled with thiol (-SH) and were commercially derived

(Eurogentec, Gent, B). The probes were linked to HRP as described in example 1.

*In situ hybridization*

A 5'-FITC labelled poly-thymide (poly-T) probe (20-mer) served as a positive control to demonstrate the presence of messenger RNA in the cells. A probe complementary to a universally conserved 16S rRNA bacterial sequence (Eub338) labelled with HRP served as a positive control, whereas a probe labelled with HRP and complementary to the sequence of probe Eub 338 (i.e. probe NonEub338) served as a negative control. Hybridization occurred at 37° C for 16 h. After hybridization, slides were washed in hybridization buffer for 20 min at 37°C and mounted in Vectashield (Vector Labs, Ca, USA)

Slides were viewed by epifluorescence microscopy on an Olympus BX-60 microscope equipped with epifluorescence illumination (HBO-100W Mercury arc lamp and NIBA (bleu) filter-set), a Canon photocamera and a fairchild CCD camera. Photomicrographs were taken on 400 ASA ECTAChrome diapositive films by the CCD camera using a 2 sec illuminations.

Example 5. Measurement of activity of single cells via total mRNA quantification by using a poly-T oligonucleotide probe. The activity of individual cells was measured by whole cell in situ hybridization of *Escherichia coli* strain JM101 .

*Culture and growth conditions.*

Cells of *Escherichia coli* strain JM101 were grown in TY-broth at 37 °C. Every 30 min, 1 ml samples were withdrawn aseptically from the culture and the samples were immediately mixed with 100 µl of 37% paraformaldehyde. Fixation of the cells occurred for 30 min at room temperature. Fixed cells were washed twice in PBS and resuspended in a mixture of PBS

and ethanol (96%) (1 : 1). Cells were stored at -20 °C for at least 1 h prior to hybridisation.

*Hybridisation*

Hybridisation was carried out by mixing 10 µl of the ethanolic fixed cell suspensions with 90 µl of hybridisation solution (20 mM Tris-HCl, pH 7.2; 0.9 M NaCl; 0.1% SDS; 0.25 ng/ml FITC-labelled oligonucleotide probe). Cells were hybridized for 16 h at 45 °C with a FITC-labelled poly-T oligonucleotide probe (20-mer, 5'-FITC), which was commercially derived.

*Probe detection.*

After hybridisation, cells were washed 4x with hybridisation buffer (hybridization solution without probe). Hybridized cells were either used immediately for flow cytometric analysis and the fluorescence was evaluated qualitatively by epifluorescence microscopy. For epifluorescence microcopy, cells were filtered onto a 0.2 mm pore-size Isopore™ polycarbonate membrane filter (Millipore Corporation, U.S.A.) and rinsed 3 x with PBS. Filters were mounted on microscope slides with Vectashield™. Hybridised cells were viewed under blue light excitation using an Olympus BX40 fluorescence microscope and epi-illumination. Flow cytometric analysis occurred at 488 nm by using a Becton Dickinson FACScalibur equipped with a 15 mW argon ion laser and a 10 mW red diode laser. TOTO I (Molecular probes Europe BV, Leiden, the Netherlands) was used as the counter stain. In all cases 10,000 cells were counted and the fluorescence from FITC was quantified. Figure 3 shows the decay in mRNA-derived fluorescence upon entering of the stationary phase of the culture.

Example 6: In situ hybridization of *Lactobacillus casei* (DSM 20011) and *Lactobacillus helveticus* (DSM 20075)

**Experimental Procedure**

Overnight cultures of *Lactobacillus casei* strain DSM 20011 and *Lactobacillus helveticus* strain DSM 20075 in MRS broth at 30°C are washed in PBS and dehydrated for 1 hour at -20C in PBS/Ethanol (1:1). Cells are pelleted by centrifugation and the supernatant is discarded. The pellet is resuspended in 300 µl of permeabilization mixture and the cells are incubated at 5°C for various periods of time, up to 180 min. The following permeabilization mixtures are tested: mixture A (see permeabilization formula of example 1), and mixture B (60 µl pronase 100 U/ml, 40 µl proteinase K 20 mg/ml). A volume of 30 microlitres of the permeabilized cell suspension is mixed with 1 ml of PBS (see example 1) and cells are pelleted by centrifugation at 14.000 rpm. The pellet is resuspended in 400 µl of PBS/Ethanol (1:1) and dehydrated at -20°C for 1 hr. Cells are pelleted by centrifugation at 14.000 rpm, the pellet is resuspended in 200 µl of hybridisation mixture (see example 5) and cells are hybridized overnight. A volume of 5 microlitres of the cell suspension is filtered over a 0.2 µm polycarbonate filter, washed with hybridization buffer and viewed under the microscope using the appropriate filtersets. Fluorescence of cells is scored visually.

**Results**

The results of the visual examinations of this study are summarised in Table 4. Clearly, the permeabilization procedure represents a controlled process by which cells become more and more permeable to the fluorophore upon prolonged incubation. Also, at a certain point, lysis starts to occur resulting in deminishing fluorescence and loss of cells. Depending on the concentration of the reactants in the mixture (and the incubation temperature, which is not

tested), the first signs of permeabilization with a standard mixture take more than 3 hours to appear in strain DSM 20011, while strain DSM 20075 can be fully permeabilized in less than 3 hours. These times are conciderably longer than those needed to permeabilize lactococcus cells (see example 1). When the concentration of the permeabilization mixture is increased 5x, permeabilization of strain DSM 20011 is already complete after 60 min of incubation at 4°C. Mixture B was derived from a study on the effect of various permeabilizing components described in example 9.

Example 7: Conventional FISH versus FISH with HRP-labeled oligonucleotide probes in the flow cytometric measurements of cells stained by 16S rRNA-FISH.

**Experimental Procedure**

*'Conventional' FISH*

Faecal bacterial samples are prepared by suspending 0.5 grams of fresh faecal material in 4.5 ml of PBS by using a dozen sterile glass beads (3 mm diameter) and a vortex. The suspension is centrifuged at low speed (1,000 rpm) for 1 min and the supernatant - which contains the cells - is transferred to a clean microcentrifuge tube. The cell suspension is then fixed in 4% *p*-formaldehyde at 4°C for 4 hrs. Cells are washed twice in PBS and resuspended in a mixture of PBS/Ethanol or another organic solvent (1:1) for dehydration and/or partial degradation. Cells are dehydrated for at least 1 hr at -20°C. Dehydrated cells are suspended one in ten in hybridization buffer and hybridized as described above without permeabilization. Hybridization was performed with probes NonEub, Eub, Bif and Uni labeled with FITC and NonEub, Eub and Bif labeled with Cy5. Probe concentrations of 2 ng/$\mu$l were used.

Washing was performed both in hybridization buffer from which SDS was ommitted and in ice-cold PBS. In the case of PBS washings, a volume of 1 ml was added to 500 $\mu$l of hybridized

cells. The cell suspension was incubated on ice for 5 min and centrifuged at 14,000 rpm for 5 min. The resulting cell pellet was resuspended in 35 µl of ice-cold PBS and stored on ice. Just prior to flow cytomerty, 5 µl of cell suspension was diluted into 500 µl of ice-cold PBS and cellular fluorescence was acquired by flow cytometry. In the case of hybridization buffer washings, a volume of 1 ml of pre-warmed (45°C) hybridization buffer without SDS was added to the hybridized cells and incubated for 30 min at 45°C. Cells were pelleted by centrifugation at 14,000 rpm for 5 min and resuspended in 1 ml of pre-warmed (45°C) hybridization buffer. The results of the FISH procedure are displayed in table 5 and in figs. 4 and 5.

*FISH with HRP probes for signal amplification.*

A volume of 50 µl of permeabilized LD-BOS cheese starter culture in PBS:Ethanol (1:1) (see example 1), representing a total of $5.10^9$ cells per ml, was pelleted by centrifugation. The cell pellet was resuspended in 100 µl of hybridization mixture consisting of 10 µl of non-purified HRP-labeled oligonucleotide (1 in 10 diluted in PEN buffer, see example 2), 8 µl of 6M ureum and 82 µl of hybridization buffer as used above, and incubated for 16 hrs at 37°C. After hybridization, cells were washed for 5 min in 1 ml of hybridization buffer with 0.48 M ureum and pelleted. Washing of the cells subsequently occurred in washing buffers of which the sodium content was gradually decreased (from 0.9 M through 0.75 M and 0.4 M to 0.15 M of NaCl). Finally the pellet was resuspended in FT substrate solution diluted to an effective substrate concentration of 35 µM of FT in PER buffer (see example 3) and incubated for 30 min at room temperature in the dark. Cells were washed twice in washing solution (100 mM Tris-HCl, pH 7,4, 150 mM NaCl, 0.05% v/v Tween 20) and the final cell pellet was resuspended in 50 µl of ice-cold PBS and stored on ice. A volume of 5 µl of this suspension was added to 500 µl of ice-cold PBS (pH 7,4) and

immediately used for flow cytometric measurement. The results of the tests with HRP labeled probes are summarized in table 6 and fig. 6.

**Results**

*'Conventional' FISH*

The results of the `conventional' FISH procedure are displayed in table 5 and figs. 4 and 5. As can be concluded from the signals derived with the FITC-labeled probe Eub - which stains virtually all bacteria - a pH of around 10.4 in the measuring buffer yields optimal fluorescence intensities. When hybridising with the *Bifidobacterium*-specific Bif probe, distinct subpopulation of cells with above-background fluorescence (*Bifidobacterium* spp.) could only be established for FITC labeled probes at pH values 9.5 and 10.4 (see fig. 4A), or when using Cy5 labeled probes (see fig. 4B). Although the staining of bifidobacteria by using the Cy5-labeled Bif probe resulted in a slightly better separation between positive (Bif labeled) and negative (non-stained) cells (see fig. 4) fluorescence intensities were overall comparable. As another optimisation of `conventional' FISH for flow cytometric application Cy5 labeled oligonucleotide probes were combined with YOPRO-1 and Cyto-9 (both derived from Molecular Probes Inc.) as general nucleic acid counter stains (see fig. 5). In this way, nucleic acid-containing particles (bacteria) could be distinguished from DNA-void bodies (detritus) prior to measuring their fluorescence. Optimal staining of the cellular nucleic acids was achieved when using Cyto-9 as a nucleic acid stain. However, although Cyto-9 (panel C) resulted in the highest fluorescence intensities (compare upper panels), YOPRO-1 at 10x diluted commercial concentration (panel A) resulted in better separation between positive and negative cells (lower panels) than YOPRO-1 at 40x diluted commercial concentration (panel B, lower panel) or Cyto9 (panel C, lower panel) (see also fig. 4

panel B, where excellent separation of the bifidobacteria is achieved in combination with YOPRO-1 at 10x diluted commercial concentration).

As a result of these tests, we concluded that procedures for `conventional' FISH were optimised and true comparison with HRP protocols was thereafter allowed.

*FISH with HRP probes for signal amplification.*

The results of the tests with HRP labeled probes are summarised in table 6 and fig. 6. The enormous increase in the intensity of fluoresceine-derived fluorescence as a result of the HRP labeling and fluoresceine-tyramide precipitation is striking. Compared to `conventional' labeling procedures, where optimisation by pH adjustment can result in a 4x increase in fluorescence (see table 5), HRP labeling results in a 30x increase in fluorescence as compared to conventional procedures (see table 6). It should be stated that the pH of the measuring buffer in these HRP experiments was 7.4 and therefor not optimal and that a further increase in signal is possible when optimal conditions - such as higher measuring pH - are used (up to 3 times). The high background fluorescence of cells as notable in test with the HRP labeled NonEub probe was due to non-specific binding and could be reduced by using blocking reagents known to the art, such as Denhardts solution. Figs. 6 demonstrates the shift in fluorescence upon labeling with HRP probes in FISH in graphical display. The intensity of HRP labeling is comparable to the staining with the nucleic acid stain YOPRO-1 at a dilution of 40x (see fig 5, panel B). These types of intensities are required for flow cytometric detection of FISH in bacterial cells, especially in cells that exist in a low level metabolic state, i.e. in which conventional FISH results in fluorescence levels too low for convenient detection such as soil bacteria or bacteria in natural water bodies.

Example 8: Detection of nisin mRNA gene-transcripts in *Lactococcus lactis*.

**Experimental Procedure**

In this experiment the procedure used in example 4 is used to detect messenger RNA's in whole cells of *Lactococcus lactis* for the antimicrobial peptide nisin. Hereto, cells of strain 161-5 of nisin producing *Lactococcus lactis* were subjected to nisin-inducing growth conditions. Samples were withdrawn every hour for 8 hrs and the cells were treated and hybridized with a HRP-labeled oligonucleotide specific for nisin-mRNA as described in example 4. A non-producing strain (strain 1614) served as a negative control in this experiment. Fluorescence was monitored visually and photographs were taken for later verification.

**Results**

The results of these tests are summarised in fig. 7. Cells of strain 1614 (fig. 7 panel A) did not show any change in the intensity of fluorescence during the course of the experiment. However, cells of strain 161-5 (fig. 7 panel B) displayed high fluorescence intensities during the first 2 hours of growth under inducing conditions and a diminishing degree of fluorescence in the following 2 hrs. After 4 hours, fluorescence returned to background levels.
This experiment shows that detection of mRNA gene-transcripts can readily be undertaken provided that appropriate permeabilization procedures are used.

Example 9: Detection of *Lactobacillus helveticus* by 16S rRNA-targeted fluorescence *in situ* hybridization.

**Experimental Procedure**

Overnight cultures of *Lactobacillus helveticus* strain DSM 20075 in MRS broth at 30°C are washed. Cells are pelleted by centrifugation and the supernatant is discarded. The pellet

is resuspended in 300 µl of permeabilizing mixture and the cells are incubated for 15 minutes at room temperature. The various permeabilization mixtures used are summarised in table 8. Following the permeabilization cells are fixed in 3.7% formaldehyde at 4°C for 30 min. The cells are washed twice in PBS, spotted to convenient densities on multi-wells microscope slides and dried at 45°C 15 min. The cells are dehydrated in a graded ethanol series (50%, 70% and 100% of ethanol) by submersing the entire slides in the various ethanol solutions for 2 min. The slides are dried to air and a volume of 50 µl of hybridization mixture with FITC labeled probes is applied to the slides. The entire slide is covered by a large cover glass, and incubated in a hybridization chamber for a period of 4 hrs. Upon hybridization, slides are washed in hybridization buffer at 45°C for 5 min, dried in air and mounted for microscopic examination.

**Results**

The results of the permeabilization experiments with *Lactobacillus helveticus* strain DSM 20075 are summarised in table 9 and fig. 8. Certain mixtures (mixtures 1 and 2) result in the immediate lysis of the cells. Such mixtures are not directly suited for controlled lysis needed to permeabilize the cells. However, lower concentrations of the compounds used in these mixtures may still yield useful results in permeabilization procedures. Clearly, for *Lactobacillus helveticus* strain DSM 20075, a mixture consisting of a combination of proteinases (mixtures 12, 16, 17 and 18) results in excellent permeabilization of *Lactobacillus helveticus* cells. Such a mixture was also used in example 6. In fig. 8, pictures of *Lactobacillus helveticus* strain DSM 20075 hybridized with probe Eub without permeabilization (panel A) and permeabilized with mixture 16, 17 or 18 consisting of a combination of proteinase K and protease type XXI (panel B) are presented.

Table 1. Bacterial cell wall components, their biochemical details and the resistance of these structures to probe penetration.

| Cellular layer | Dominant macromolecules | Chemistry | Penetration resistance | Degrading component |
|---|---|---|---|---|
| Lipopolysaccharide | LPS (polysaccharides and lipids) | sugar / lipid (fatty acid and phosphate) | surface coverage | finizym / lipase+ taurocholate |
| phospholipid | | lipid | surface polarity | lipase+ taurocholate |
| peptidoglycan (mucopeptide) | *N*-acetylglucosamines (NAG) *N*-acetylmuramic acids (NAM) tetrapeptides (lipo)teichoic acids and teichuronic acids | sugar / peptide sugar / peptide peptide sugar / lipid | structura l barrier structura l barrier structura l barrier | lysozyme finizym, $Ca^{2+}$ /lipase+ taurocholate autolysins |
| cytoplasmic membrane | phoshatidylethanolamines and phosphatidylglycerol | lipid | surface polarity | lipase+ taurocholate |

Table 2. Bacterial cell wall components, biochemical details and resistance to probe penetration.

| Component | Function |
|---|---|
| saccharose (sucrose) or lysine betaine | Osmoticum, prevents cells from bursting |
| sodium taurocholate | detergent, increases surface area of lipids |
| calcium chloride | Decreases cell wall rigidity by competing with bivalent cations that keep in check the electronegative repulsion from teichoic and teichuronic acids |
| Tris-HCL | buffer |
| lysozyme | glycosidase that splits disaccharide bond between *N*-acetylglucosamines (NAG) and *N*-acetylmuramic acids (NAM) thereby degrading peptidoglycan |
| lipase | degrades lipids by hydrolysing ester-bond between glycerol moiety and fatty acid tails |
| finizym | beta-glucanase that hydrolyses 1,4- and 1,3-beta-glucan bonds thus producing oligosaccharides and disaccharides |
| mutanolysin | degrades *Streptococcus faecalis* cell walls |

Table 3.

Probes for DNA and mRNA of proteinase of *Lactococcus lactis* subspecies *lactis*

| | | |
|---|---|---|
| PRT1245: | 5'-GCCCCTAAAGCGACTGTACC-3' |
| PRT4548: | 5'-GATACCGCCGCCGTTCACA-3' |
| PRT6497: | 5'-TGTCAGACGCATCAGCACTG-3' |

Probes for *Lactobacillus casei* 16S rRNA

| | | |
|---|---|---|
| LBC186 | 5'-TTCAGCCAAGAACCATGCG-3' |
| LBC194 | 5'-CTTACGCCATCTTTCAGCCA-3' |
| LBC446 | 5'-ACGCCGACAACAGTTACTC-3' |
| LBC453 | 5'-CTCTGCCGACCATTCTTCT-3' |
| LBC1000 | 5'-CCTGATCTCTCAGGTGATC-3' |
| LBC1021 | 5'-GCCCCCGAAGGGGAAAC-3' |
| LBC1008 | 5'-GGAAACCTGATCTCTCAGG-3' |
| LBC1448 | 5'-GGCTCGCTCCCTAAAAGG-3' |

Probe for *Lactococcus lactis* subspecies *cremoris* 23S rRNA

| | | |
|---|---|---|
| LLC1199 | 5'-CTCCTACCATTGATAAAATATCAAT-3' |
| LLC1202 | 5'-CTACCATTGATAAAATATCA-3' |

Probe for *Lactococcus lactis* subspecies *lactis* 23S rRNA

| | | |
|---|---|---|
| LLL1199 | 5'-CTCCTACCATTTAATTAAATTAAAT-3' |
| LLL1202 | 5'-CTACCATTTAATTAAATTAA-3' |

Table 3, continued

Probes for *Mycobacterium avium* subspecies *paratuberculosis* 23S rRNA

| | | |
|---|---|---|
| MPA748 | 5'-CGCCACTACACCCCAAAAG-3' |
| MPA1320 | 5'-GATCACCCGGAGCTTCG-3' |
| MPA1705 | 5'-GTGACGGACGGATTTGCC-3' |
| MPA1712 | 5'-CAGGATTAGTGACGGACGG-3' |
| MPA1796 | 5'-GGCAGTGTATGTGCTCGC-3' |
| MPA2760 | 5'-CGAACAGCCCAACCCTTG-3' |
| MPA3029 | 5'-TGATCTGCGGGGGGCCT-3' |

Probe for *Mycobacterium avium* subspecies *paratuberculosis* ITS sequence (Internally Transcribed Spacer between 16S and 23S rRNA codons)

| | |
|---|---|
| MPAITS | 5'-AACGCCGGCCACGGAGA-3' |

Probes for "Dermatitis Digitalis" related *Treponema* species 16S rRNA

| | |
|---|---|
| TDD141 | 5'-CGGATACCCATCGTYGCC-3' |
| TDD154 | 5'-TCTAGSAGCTATCCCCATC-3' |
| TDD275 | 5'-ATTCGGTATTACCTSCTATTTC-3' |

Probes for *Clostridium difficile* 16S rRNA

| | |
|---|---|
| CDI174 | 5'-CTCTCAAATATATTATCCCGT-3' |
| CDI190 | 5'-CACCTTTGATATTCAAGAGA-3' |
| CDI198 | 5'-ATCCTGTACTGGCTCACC-3' |
| CDI825 | 5'-GTAACCCCCGAACACCTAGT-3' |
| CDI845 | 5'-ACGTGCGGCACCGAGGGGGG-3' |

Table 4. Effect of permeabilization mixture on hybridization efficiencies for the detection of *Lactobacillus* species (results for example 6).

| Permeabilization time | Probe | Mixture A: Feacal sample | Mixture A: Lactobacillus helveticus DSM 20075 | Mixture A: Lactobacillus casei DSM 20011 | Mixture A (5X): Lactobacillus casei DSM 20011 | Mixture B: Feacal sample | Mixture B: Lactobacillus helveticus DSM 20075 | Mixture B: Lactobacillus casei DSM 20011 |
|---|---|---|---|---|---|---|---|---|
| 15 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | + >95% | + >95% | + <1% | | ++ >95% | ++ >95% | + 10% |
| | Lab | + <5% | + 15% | + <1% | | + 30% | ++ 50% | + 10% |
| 30 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | ++ 10% | ++ 60% | ++ <1% | | ++ 40% | ++ 70% | + 5% |
| | Lab | - 100% | ++ 50% | ++ <1% | | +/- 5% | ++ 50% | + 5% |
| 60 min | NonEub | - 100% | - 100% | - 100% | - 100% | - 100% | - 100% | - 100% |
| | Eub | ++ 75% | ++ 70% | + 2% | ++ 98% | ++ 85% | ++ 70% | + 10% |
| | Lab | - 100% | ++ 50% | + 2% | ++ 95% | + 5% | ++ 50% | + 10% |
| 90 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | ++ 50% | ++ 85% | + 2% | | ++ 70% | ++ 90% | + 7% |
| | Lab | - 100% | ++ 55% | + 2% | | +/- n.d. | ++ 55% | + 7% |
| 120 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | - 100% | + 70% | + 4% | | ++ 95% | ++ >95% | + 8% |
| | Lab | - 100% | + 65% | + n.d. | | - 100% | ++ 65% | + 10-12% |
| 150 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | ++ 10% | + 60% | + 5% | | ++ 80% | ++ 90% | ++ 7% |
| | Lab | - 100% | + 50% | + 5% | | - 100% | ++ 90% | + 7% |
| 180 min | NonEub | - 100% | - 100% | - 100% | | - 100% | - 100% | - 100% |
| | Eub | ++ 25% | + 80% | + n.d. | | ++ 60% | ++ 95% | + 10% |
| | Lab | - 100% | + 50% | + <20% | | +/- n.d. | ++ 95% | + 20% |

Table 5. Effect of label and pH on the fluorescence intensity by 'conventional' FISH of bacteria in human intestinal microflora as determined by flow cytometry.

| | FITC | | | | | | | | Cy5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH 7.4 | | | pH 9.4 | | | pH 10.4 | | pH 7.4 | |
| Probe | Mean | Median | | Mean | Median | | Mean | Median | Mean | Median |
| Non-Eub | 3,92 | 1,83 | | 5,88 | 2,79 | | 4,90 | * 2,23 | 53,21 | 12,19 |
| Bif | n.d. | n.d. | | 12,10 | 4,74 | | 12,05 | 4,87 | 35,93* | 7,57 |
| Eub | 12,90 | 7,37 | | 43,96 | 21,87 | | 48,80 | 24,14 | 62,73 | 33,68 |
| Uni | 9,16 | 4,83 | | 33,11 | 16,25 | | 31,19 | 15,40 | n.d. | n.d. |
| Uni + Eub | 19,76 | 10,00 | | 67,14 | 31,91 | | 80,82 | 38,54 | n.d. | n.d. |

* Percentage of cells with above-background fluorescence (*Bifidobacteria*): 7,20%

Table 6. Effect of HRP labelling on the fluorescence intensity of *FISH* in LD-BOS cheese starter culture as determined by flow cytometry.

| Probe | FITC | | | HRP-FITC | |
|---|---|---|---|---|---|
| | Mean | Median | | Mean | Median |
| Non-Eub | 4,44 | 2,18 | | 80,87 | 39,79 |
| Eub | 15,80 | 8,45 | | 468,80 | 135,77 |
| LLC | 17,63 | 9,38 | | 549,48 | 198,90 |

n.b. The pH of the measuring buffer was 7.4 in these experiments.

Table 7. 16S rRNA Probes used in this study

| Probe Name | Specificity | Sequence (5'-3') | Labels |
|---|---|---|---|
| Non-Eub | negative control probe | ACTCCTACGGGAGGCAGC | FITC, Cy5, HRP |
| Eub | Vitually all Bacteria | GCTGCCTCCCGTAGGAGT | FITC, Cy5, HRP |
| Bif | Genus *Bifidobacterium* | CATCCGGCATTACCACCC | FITC, Cy5 |
| LLC | *Lactococcus lactis* subsp. *cremoris* | TGCAAGCACCAATCTTCATC | FITC, Cy5, HRP |
| Uni | Universal (Eukaryae, Archaea and Bacteria) | G(AT)ATTACCGCGGC(GT)GCTG | FITC, Cy5 |
| Lab | Genus *Lactobacillus* | GGTATTAGCA(TC)CTGTTTCCA | FITC |

## Table 8. Composition of Permeabilization Mixtures used in example 9.

| | |
|---|---|
| 'Mixture' 0 | - milli-q 300 µl |
| Mixture 1 | - polymyxine 80 µl (of a 0,00075 g/ml solution)<br>- streptomycine 40 µl (of a 0,003 g/ml solution)<br>- lipase 160 µl (of a 1 mg/ml)<br>- $CaCl_2$ 200 µl (of a 22,2%)<br>- Lysozym 80 µl (of a 200 mg/ml solution)<br>- mutanolysine 40 µl (of a 10.000 U/ml solution)<br>- finizym 40 µl<br>- milli-q 560 µl |
| Mixture 2 | - polymyxine 80 µl (of a 0,00075 g/ml solution)<br>- streptomycine 40 µl (of a 0,003 g/ml solution)<br>- lipase 160 µl (of a 1 mg/ml solution)<br>- $CaCl_2$ 200 µl (of a 22,2% solution)<br>- Lysozym 80 µl (of a 200 mg/ml solution)<br>- mutanolysine 40 µl (of a 10.000 U/ml solution)<br>- milli-q 600 µl |
| Mixture 3 | - lysozym 20 µl (of a 200 mg/ml solution)<br>- mutanolysine 10µl (of a 10.000 U/ml solution)<br>- finizym 10 µl<br>- milli-q 260 µl |
| Mixture 4 | - polymyxine B 20 µl (of a 0,00075 g/ml solution)<br>- streptomycine 10µl (of a 0,003 g/ml solution)<br>- lipase 40 µl (of a 1 mg/ml solution)<br>- $CaCl_2$ 50 µl (of a 22,2 % solution)<br>- finizym 10 µl<br>- milli-q 170 µl |
| Mixture 5 | - toluene 3 µl<br>- milli-q 297 µl |
| Mixture 6 | - toluene 30 µl<br>- milli-q 270 µl |
| Mixture 7 | - DIDS 150 µl (of a 2mg/ml solution)<br>- milli-q 150 µl |
| Mixture 8 | - DTT 150 µl (of a 1M solution)<br>- milli-q 150 µl |
| Mixture 9 | - DIDS 150 µl (of a 2 mg/ml solution)<br>- DTT 150 µl (of a 1M solution)<br>- Toluene 30µl |
| Mixture 10 | - LiCl 300 µl (of a 5M solution) |
| Mixture 11 | - LiCl 270 µl (of a 5M solution)<br>- lysozyme 20 µl (of a 200 mg/ml solution)<br>- mutanolysine 10 µl (of a 10.000 U/ml solution) |
| Mixture 12 | - proteinase K 20 µl (of a 20 ng/ml)<br>- pronase (protease type XXI) 30 µl (of a 100 U/ml solution)<br>- milli-q 250 µl |
| Mixture 13 | - LiCl 300 µl (of a 10M solution) |
| Mixture 14 | - LiCl 300 µl (of a 15M solution) |
| Mixture 15 | - LiCl 270 µl (of a 10M solution)<br>- lysozyme 20 µl (of a 200 mg/ml solution)<br>- mutanolysine 10 µl (of a 10.000 U/ml solution) |
| Mixture 16 | - proteinase K 20 µl (of a 20 ng/µl solution)<br>- pronase 30 µl (of a 100 U/ml solution)<br>- milli-q 250 µl |
| Mixture 17 | - proteinase K 40 µl (of a 20 ng/µl solution)<br>- pronase 60 µl (of a 100 U/ml solution)<br>- milli-q 200 µl |
| Mixture 18 | - proteinase K 60 µl (of a 20 ng/µl solution)<br>- pronase 90 µl (of a 100 U/ml solution)<br>- milli-q 150 µl |
| Mixture 19 | *Permeabilisation formula (see example 1):* |

Table 9. Effect of different mixtures on the permeabilization of Lactobacillus helveticus strain DSM 20075 for FISH.

| | Probe | | |
|---|---|---|---|
| Mixture | NonEub | Eub | Lab |
| 0 | - | - | - |
| 1 | lysis | lysis | lysis |
| 2 | lysis | lysis | lysis |
| 3 | - | - | n.d. |
| 4 | - | - | n.d. |
| 5 | - | - | n.d. |
| 6 | - | - | n.d. |
| 7 | - | - | n.d. |
| 8 | - | - | n.d. |
| 9 | - | - | n.d. |
| 10 | - | + | +/- |
| 11 | - | + | n.d. |
| 12 | - | ++ | n.d. |
| 13 | - | + | +/- |
| 14 | - | + | +/- |
| 15 | - | + | +/- |
| 16 | - | + | +/- |
| 17 | - | ++ | + |
| 18 | - | +++ | ++ |
| 19 | - | + | +/- |

- : no fluorescence
-/+ : weakly fluorescent
+ : properly fluorescent
++ : very good fluorescence
+++: bright fluorescence

n.d., not determined

Figures

Figure 1. Illumination series demonstrating the enormous (80x) increase in fluorescence emission from HRP-labelled probe (photograph D: 0.5 sec illumination) compared to conventional FITC-labelled oligonucleotide probes (photographs A, B and C: 0.5, 4 and 10 sec illumination, respectively). Hybridizations were performed on cheese starter-culture LD-Bos. The HRP-labelled probe used was targeted to the 23S rRNA of *Lactococcus lactis* subspecies *cremoris* cells (over 50%). The FITC-labelled probe was targeted to the 16S rRNA of all bacterial cells present in the sample. The protocol for in situ hybridization with HRP-labelled probes of example 1 was applied. The average fluorescence of cells in photograph D was 181, that of photograph C was 53 (in AU) as determined by image cytometry.

Figure 2. CCD-images of the *prt+* strain of *L. Lactis* spp. *cremoris* str. MG1363 (NCDO 736) hybridized with a mixture of the three PRT probes as described in example 2. Comparison of phase contrast (A) and fluoresceine fluorescence (B) images show that the protease transcript is not present in equal amounts in individual cells.

Figure 3. Measurement of mRNA via an FITC-labelled poly-T oligonucleotide probe in individual Escherichia coli cells by flow cytometry.

Figure 4

Flow cytometer cytograms representing the measurements of human intestinal (faecal) flora with *Bifidobacterium* genus-specific 16S rRNA targeted *in situ* hybridization probes labeled at the 5'end with FITC (panel A) and Cy5 (panel B). Fluorescence intensities (y-axis) are plotted versus Forward Scattering Signal (x-axis). With both labels, a distinct group of cells with higher fluorescence than the body of the

population can be distinguished. Pthe pH of the measuring medium was 10.4 for FITC and 7.5 for Cy5 with TOPRO-3 and YOPRO-1 as the DNA counter stains for FITC and Cy5, respectively.

Figure 5
The effect of DNA counter stains on the fluorescence intensity derived from FISH. Flow cytometer histograms representing the measurements of human intestinal (faecal) flora with *Bifidobacterium* genus-specific 16S rRNA targeted *in situ* hybridization probes labeled at the 5'end with Cy5. The nucleic acid-counterstains YOPRO-1 and Cyto9 were used. Panel A, top: The YOPRO-1 fluorescence (x-axis) versus the cell counts. YOPRO-1 was applied as a 10x diluted commercial preparation. Panel A, bottom; The Cy5 fluorescence derived from these cells (x-axis) versus the cell counts. Panel B, top: The YOPRO-1 fluorescence (x-axis) versus the cell counts. YOPRO-1 was applied as a 40x diluted commercial preparation. Panel B, bottom; The Cy5 fluorescence derived from these cells (x-axis) versus the cell counts. Panel C, top: The Cyto-9 fluorescence (x-axis) versus the cell counts. Panel C, bottom; The Cy5 fluorescence derived from these cells (x-axis) versus the cell counts.

Figure 6
The effect of HRP labeling on the fluorescence intensity derived from FISH. Flow cytometer histograms representing the measurements of LD-BOS cheese starter culture with a *Lactococcus lactic* subspecies *cremoris* -specific 23S rRNA targeted *in situ* hybridization probe (LLC) labeled at the 5'end with HRP (panel B). No nucleic acid-counterstains were used. Panel A, top: The FITC fluorescence derived from HRP-labeled probe NonEub used in combination with FT-substrate (x-axis) versus the cell counts. Panel A, bottom; The far-red fluorescence derived from these cells (x-axis) versus the cell counts. Panel B, top: The FITC fluorescence derived from

HRP-labeled probe LLC used in combination with FT-substrate (x-axis) versus the cell counts. Panel B, bottom; The far-red fluorescence derived from these cells (x-axis) versus the cell counts.

Figure 7.
Nisin mRNA detection in *Lactococcus lactis*. Panel A: Non nisin-producing strain 1614 after 3 hrs of growth under nisin-inducing conditions. Panel B: Nisin-producing strain 161-5 at various times during 6 hrs of growth under nisin-inducing conditions. See text for details.

Figure 8
The *in situ* hybridization of *Lactobacillus helveticus* strain DSM 20075 with FITC-labeled probes without (panel A) and with permeabilization (panel B). Left-hand side images represent FITC fluorescence, right hand side images are corresponding phase-contrast pictures. Upon effective permeabilization, the cells become almost transparent.

## CLAIMS

1. A method for *in situ* staining of micro-organisms comprising: a) mixing a material containing at least one (fixed or non-fixed) micro-organism with a composition which can (partly) degrade a cell wall or cell membrane of a micro-organism thereby allowing for penetration through said wall and/or membrane of a (labelled) probe into said micro-organism, b) optionally fixing said micro-organism with a fixative to further retain its individual corpuscular character, c) reacting said probe with an antigen or nucleic acid molecule present in said micro-organism, and d) detecting the presence of said probe in said micro-organism.

2. A method according to claim 1 wherein said micro-organism belongs to any of the group of viruses, bacteria, yeast, fungi and plant cells.

3. A method according to claim 1 or 2 wherein said composition at least comprises one detergent, such as sodium taurocholate and at least one enzyme, such as a lipase.

4. A method according to claim 1 or 2 wherein said composition at least comprises a proteinase.

5. A method according to claim 4 wherein said proteinase is proteinase K and/or protease type XXI.

6. A method according to claims 3 to 5 wherein said composition additionally comprises a carbohydrate degrading enzyme such as lysozyme, finizym, or mutanolysin.

7. A method according to anyone of claims 3 to 6, wherein said composition additionally comprises at least one osmoticum, such as saccharose, sucrose, or lysine betaine, and/or at least one cation, such as $Ca^{++}$ or $Li^{+}$.

8. A method according to any of claims 1 to 7, wherein said fixative is selected from any of the group composed of (para)formaldehyde, glutaraldehyde, ethanol, methanol.

9. A method according to any of claims 1 to 8 wherein said probe is a nucleic acid probe.

10. A method according to claim 9 wherein said probe is directed against genomic or ribosomal DNA or against messenger or ribosomal RNA.

11. A method according to any of claims 9 or 10 wherein said probe is labelled to a reporter molecule.

12. A method according to claim 11 wherein said reporter molecule is selected from any of the group of fluorochromes or enzymes.

13. A method according to claim 12 wherein the reporter molecule is horseradish-peroxidase specifically linked, preferably with a sulfo-SMCC linker, to a nucleic acid probe.

14. A method acccording to any of claims 1 to 13 wherein the presence of said probe or its reaction products is detected by microscope, image cytometry, fluorometry or flow cytometry.

15. A method according to claim 14 wherein the presence of said probe is detected by detecting fluorescence.

16. A method according to claim 15 wherein the fluorescence of tyramine-fluorochromes is detected.

17. A method according to any of claims 13 to 15 wherein the micro-organism is not immobilised to a slide or filter.

18. A composition comprising a cell-wall degrading reagens for use in *in situ* staining which comprises at least one detergent, such as sodium taurocholate and at least one enzyme, such as a lipase, or at least a proteinase.

19. A composition according to claim 18 which further comprises a carbohydrate degrading enzyme such as lysozyme, finizym, or mutanolysi

20. A composition according to claim 18, or 19 which further comprises at least one osmoticum, such as saccharose, sucrose, or lysine betaine, to regulate

osmotic pressure and/or at least one cation, such as $Ca^{++}$ or $Li^{+}$.

21. A nucleic acid probe derived from a thiolated probe linked to horseradish-peroxidase with a sulfo-SMCC linker.

22. A method for linking a nucleic acid probe to horseradish peroxidase comprising mixing a thiol oligonucleotide with a SMCC-HRP complex, incubating said mixture and purifying it by ultrafiltration.

23. A diagnostic test kit for use with *in situ* staining which at least comprises a cell wall-degrading composition according to any of claims 18 to 20 and/or a nucleic acid probe according to claim 21.

24. An enzyme-linked assay or diagnostic kit which comprises a nucleic acid probe according to claim 21.

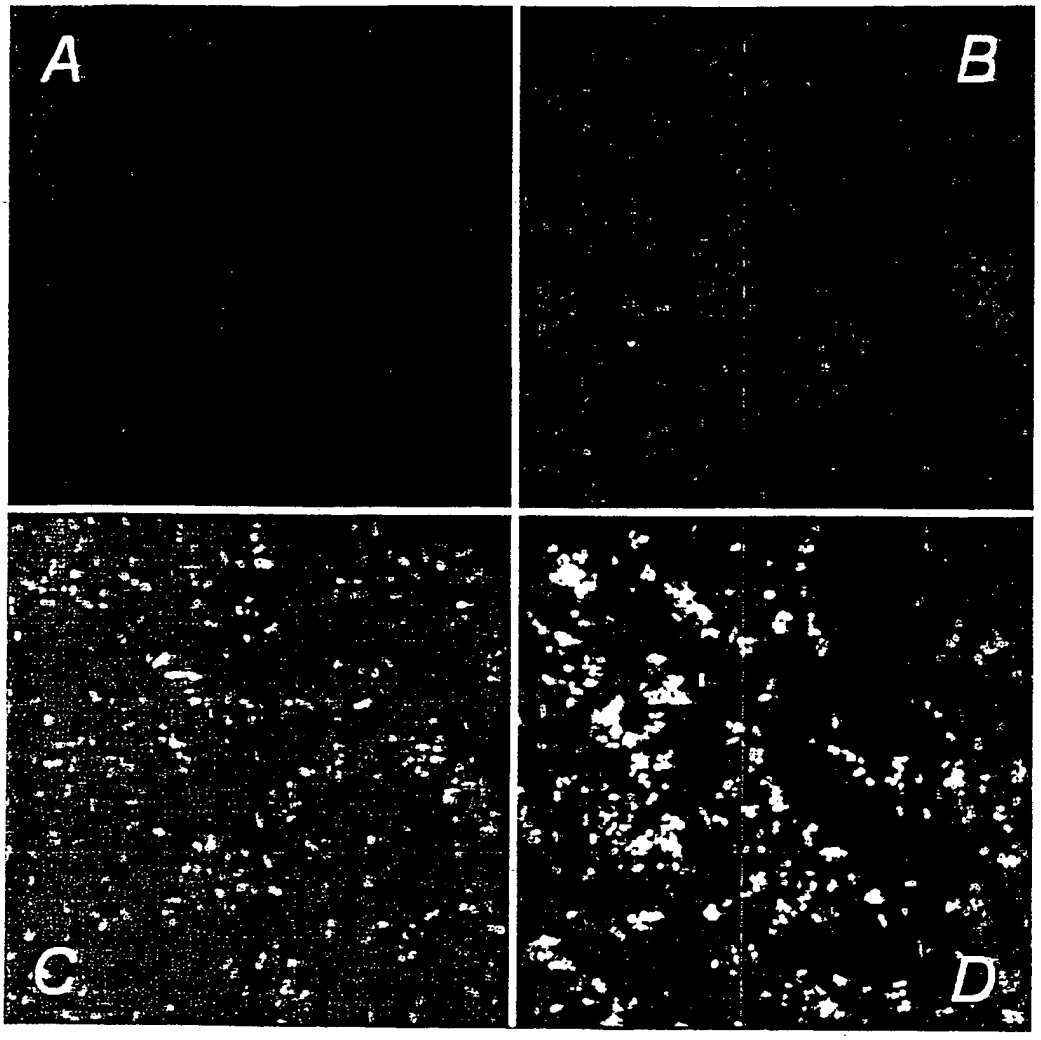



Figure 1

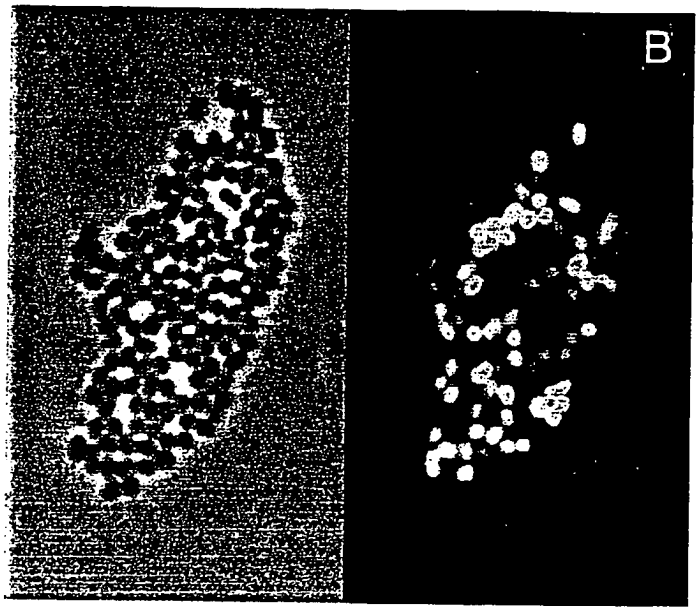



Figure 2

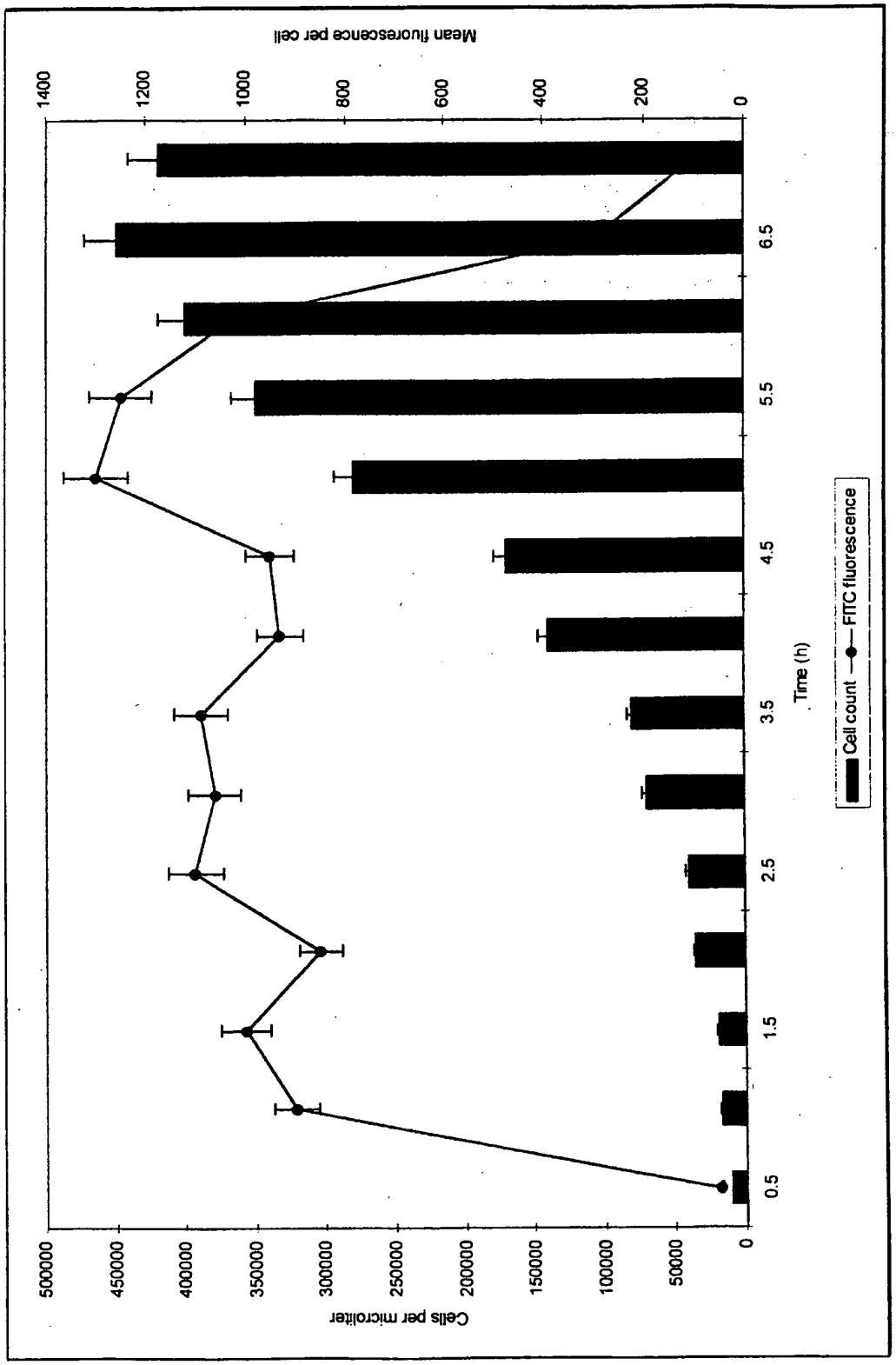
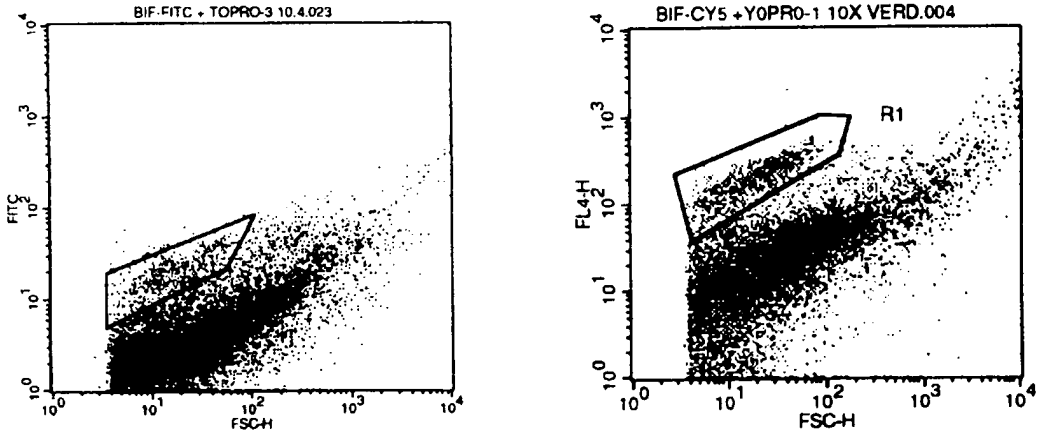

Mean fluorescence per cell
1400
1200
1000
800
600
400
200
0
Cells per microliter
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0.5
1.5
2.5
3.5
4.5
5.5
6.5
Time (h)
Cell count
FITC fluorescence

Figure 3

# Fig. 4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FITC
FSC-H
A
BIF-CY5 +Y0PR0-1 10X VERD.004
R1
FL4-H
FSC-H
B

Fig. 5

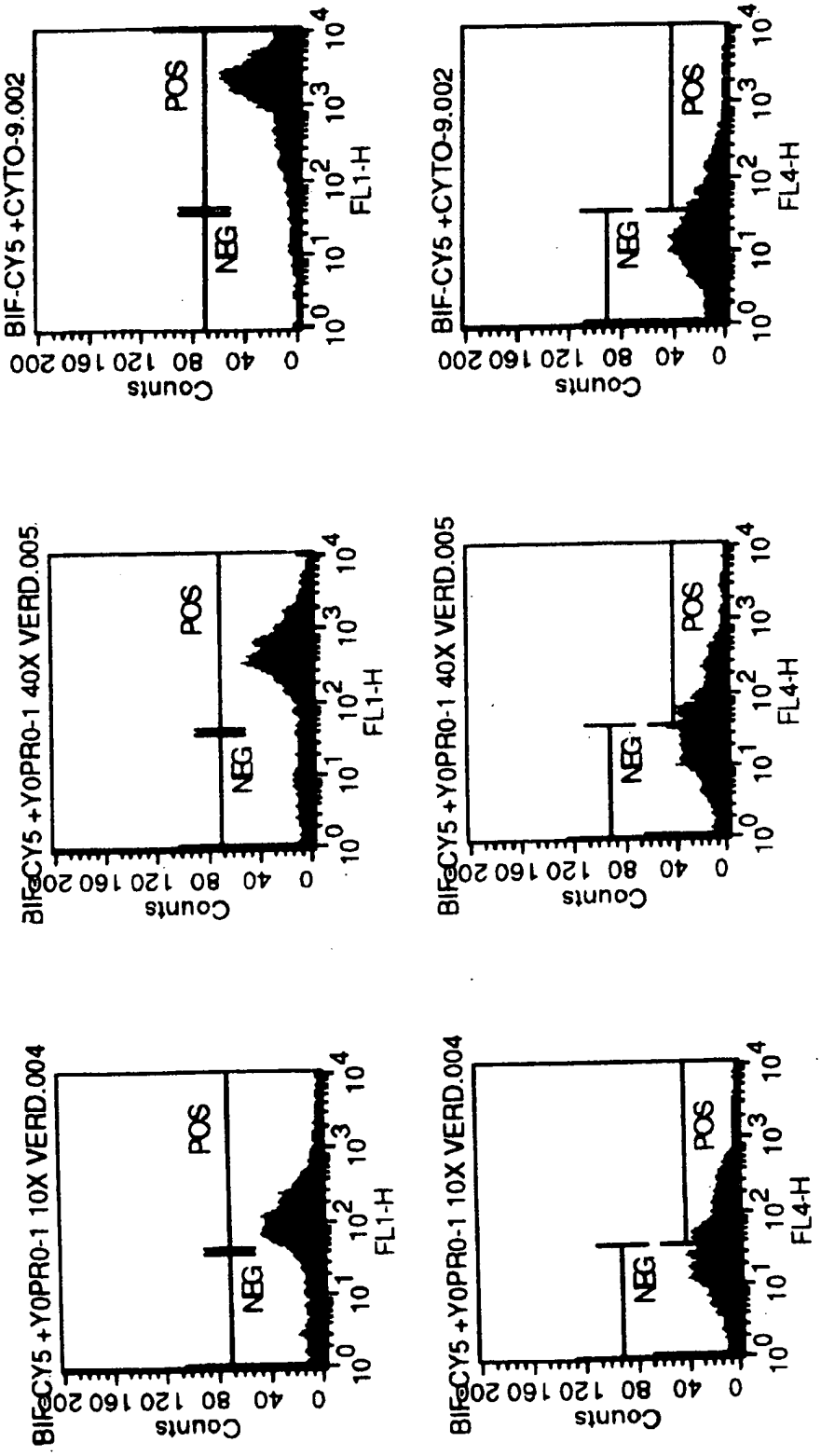
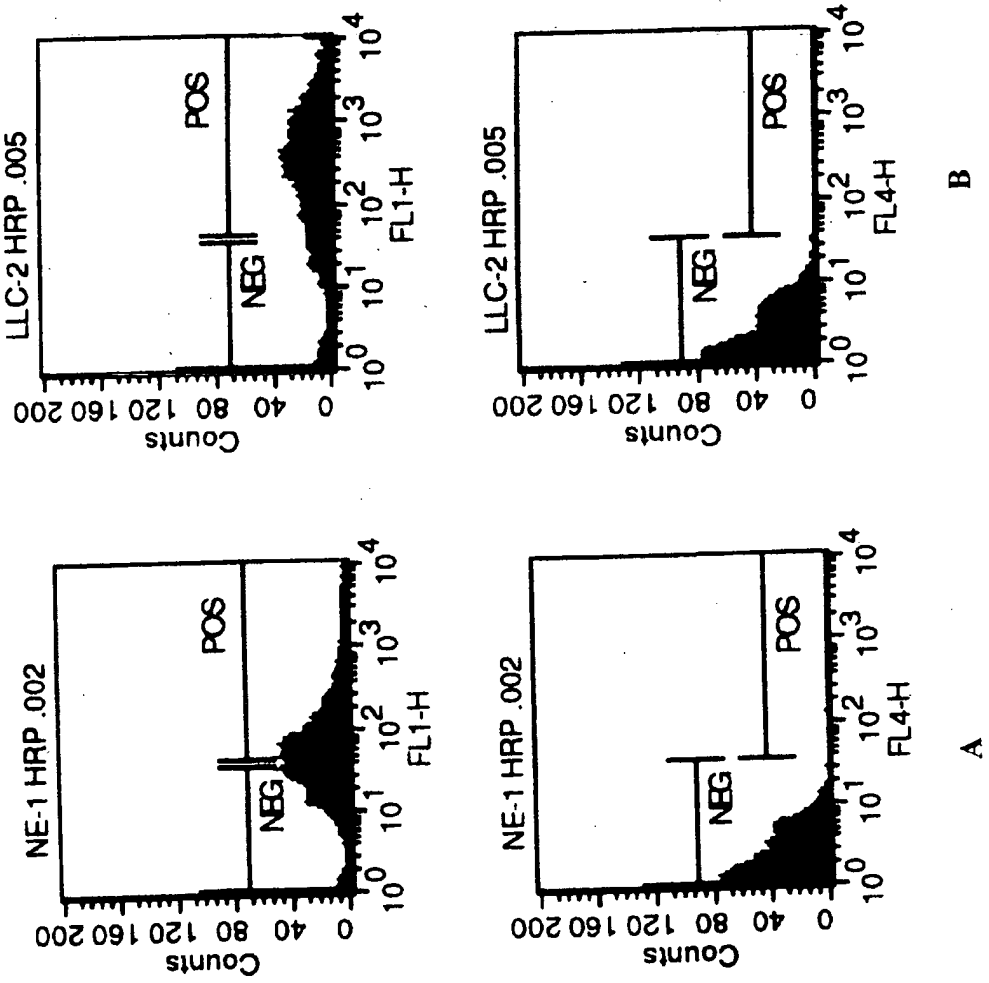

BIF-CY5 +CYTO-9.002
BIF-CY5 +Y0PR0-1 40X VERD.005
BIF-CY5 +Y0PR0-1 10X VERD.004
POS
NEG
Counts
FL1-H
FL4-H
A
B
C

Fig. 6



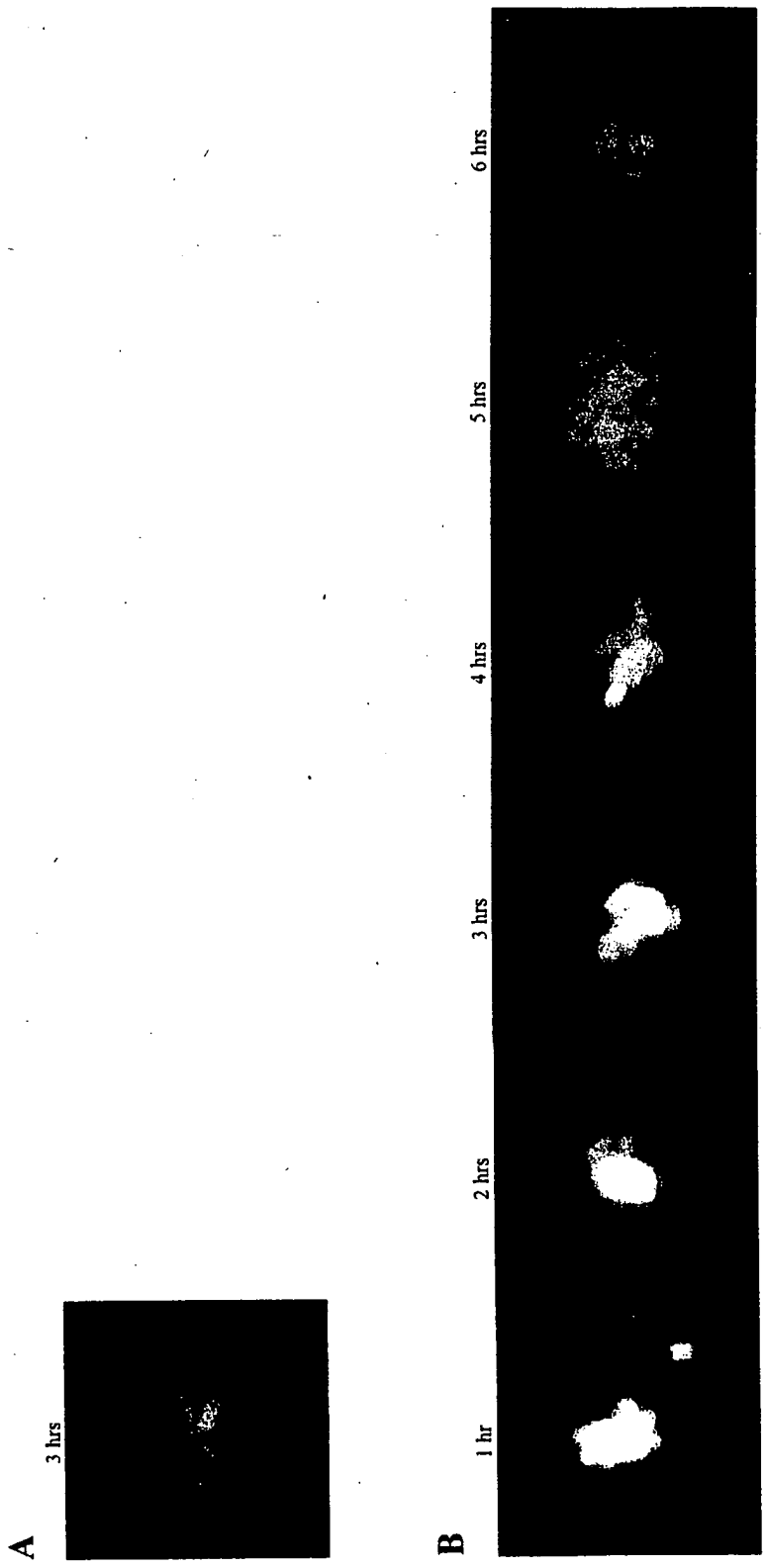
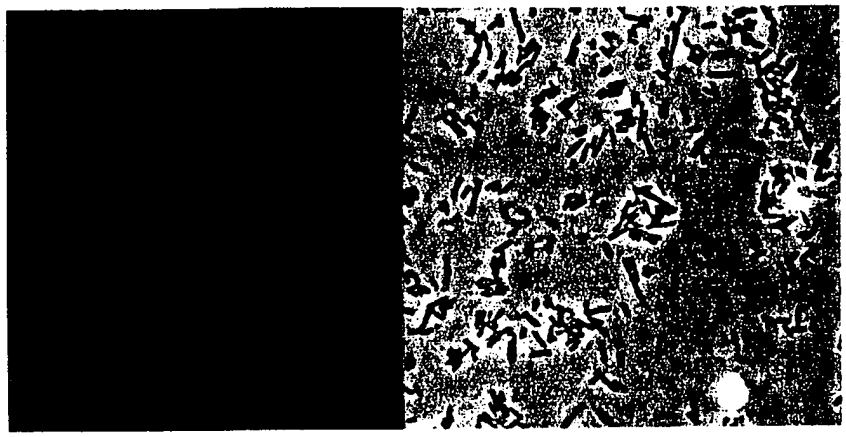
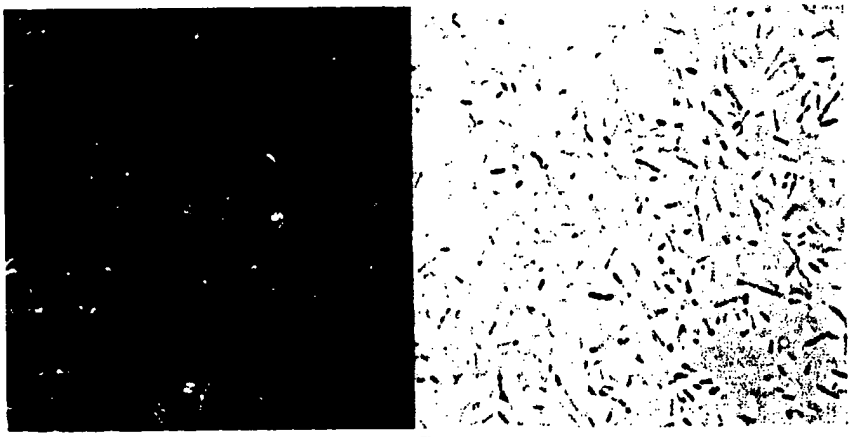



Fig. 7

# Fig. 8



A



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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 98/00481

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N33/569 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 9 December 1998 | 15/12/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Osborne, H |

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Int ational Application No
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